REMARKS

Claims 153-204 are currently pending in the present application.

Claims 153, 168, 183 and 194 have been amended to require that the sugar cane-derived extract include one or more non-saccharides as an active ingredient. Support for this amendment can be found, for example, in examples 3-4 of the present specification.

Specifically, in Example 3 of the present application, the infection-preventing effect of the less-saccharide fraction prepared in Preparation Examples 5 and 7 against E. coli of intake was tested. The groups treated with the less-saccharide fraction clearly showed higher survival ratios, compared to the control group treated with sterilized distilled water. The survival ratio increased with the increasing amount of the less-saccharide fraction. In Example 4 of the present application, the anti-viral effect of intake of the less-sugar fraction prepared in Preparation Examples 5 to 7 was tested. The groups treated with the extracts having a higher non-sugar content showed particularly high survival ratios, compared to the control group treated with sterilized distilled water.

Therefore, Examples 3-4 demonstrate that an active component of the sugar cane-derived extracts of the invention can be one or more non-saccharides.

The 35 U.S.C. 112 Rejections

Claims 153-204 have been rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. Specifically, the Examiner alleges that the phrase, "providing a remedial effect for a disease" is not described in the specification. This rejection is respectfully traversed and reconsideration is requested for the reasons that follow.

In the specification, page 8, lines 2 to 3, there is the description, the "remedial effect is that against viruses or bacteria causing infectious diseases". (emphasis added). Also, on page 23, lines 20 to 25, there is the description, "the present invention may be applied to prevent or remedy diseases caused by weakness or deficiency of immunological function through control of immunological function of man or animals. The present invention may be applied also to prevent or remedy various kinds of infectious disease."(emphasis added).

These portions of the specification therefore support the term, "providing a remedial effect for a disease", seen in claims 153 to 204. Favorable consideration and withdrawal of the rejection is requested.

Claims 153-204 have also been rejected under 35 U.S.C. 112, first paragraph on the basis that the specification does not reasonably provide enablement for treating any disease caused by any bacteria, fungus or virus. This rejection is respectfully traversed and reconsideration is requested for the reasons that follow.

The Examiner has taken the position, in support of this rejection, that "The level of predictability in treating infectious diseases is low, particularly regarding bacterial and viral infections such as anthrax, hepatitis, influenza, Ebola and HIV." See page 3, lines 6-8 of the Office Action dated August 24, 2004. While this may be true, it does not apply to the present invention because the mechanism of action of the present invention is control of immunological function. Moreover, the present invention does not treat a disease by developing specific antibodies against that disease, like, for example, a vaccine, but rather, provides a remedial effect against a disease by control of immunological function. Since this is the case, a skilled person would expect that the present invention would be generally applicable to a wide variety of diseases since it controls immunological function rather than addressing a specific disease in a disease-specific way.

In the specification on page 23, lines 20 to 25, there is a description to the effect that a sugar cane-derived extract of the invention may be applied to remedy diseases caused by weakness or deficiency of immunological function through control of immunological function of man or animals. Thus, the remedial effect is attributable to the control of immunological function. Test Examples 2, 3 and Examples 1, 2 of the specification clearly support this statement, as explained below.

In Test Example 2, the antibacterial activity of the sugar cane-derived extract against E. coli was tested in vitro. The minimum growth inhibiting concentration was found to be 10,000 microgram/ml, with the conclusion that no strong antibacterial activity was observed in vitro, as set forth on page 41, lines 21 to 22 of the specification. On the other hand, in Example 1 of the specification, a solution or suspension of the sugar cane-derived extract was orally administered to mice, on the day before inoculation of E. coli. Then, a suspension of man-origin E. coli was subcutaneously inoculated into the mice. The group treated with the sugar cane-derived extract clearly showed higher survival ratios, compared to the control groups that received sterilized distilled water. The survival ratio also increased as the administered amount of the sugar cane-derived extract increased.

Accordingly, Test Example 2 demonstrated that the sugar cane-derived extract was not an effective anti-bacterial in vitro, but Example 1 of the specification clearly demonstrated that the sugar cane-derived extract was effective in vivo. Therefore, it is seen that the sugar cane-derived extract of the invention affects an immunological function of mice since this is the explanation that fits the experimental data of these examples.

Also in Test Example 3, the proliferation-inhibiting properties of the sugar cane-derived extract against Coxsackie virus and Herpes simplex virus were tested in vitro. It was found that the sugar cane-derived extract does not have a proliferation-inhibiting effect against Coxsackie virus, and has only a weak proliferation-inhibiting effect against Herpes simplex virus even at the higher concentrations of the extract, as set forth on page 43, lines 11 to 15 of the specification. On the other hand, in Example 2 of the specification, a solution or suspension of the sugar cane-derived extract was orally or intramuscularly administered to mice, 3 times in total, i.e., immediately after, one day after and two day after the inoculation of virus, or 9 times in total, i.e. three times per day for 3 consecutive days. The groups treated with the sugar cane-derived extract clearly showed higher survival ratios, compared to the control group, which received sterilized distilled water. The survival ratio also increased with an increasing administered amount of the extract. Accordingly, these examples also demonstrate that the sugar cane-derived extract was not effective in vitro, but was effective in vivo. Therefore, it is again seen that the sugar cane-derived extract of the invention affects an immunological function of mice.

In addition, the following three articles also support the conclusion that the sugar cane-derived extracts of the invention have immuno-stimulating effects. One of the inventors, Kenji Koge, is an author of each of the three articles. Articles 2 and 2' are related to one another as the original article and the erratum for the original article.

Article 1: Moshira EL-ABASY et al. "Protective Effects of Sugar Cane Extracts (SCE) on Eimeria tenella Infection in Chickens", J. Vet. Med. Sci. 65(8): 865-871, 2003.

Article 2: Moshira El-Abasy et al. "Preventive and therapeutic effects of sugar cane extract on cyclophosphamide-induced immunosuppression in chickens", International Immunopharmacology, 4, pp. 983-990, 2004.

Article 2': Moshira El-Abasy et al. "Erratum to "Preventive and therapeutic effects of sugar cane extract on cyclophosphamide-induced immunosuppression in chickens" [International

Immunopharmacology 418 (2004), pp. 983-990], International Immunopharmacology, 4, 1565-1568, 2004.

Article 3: Said Amer et al. "Immunostimulating effects of sugar cane extract on X-ray radiation induced immunosuppression in the chicken", International Immunopharmacology, 4, 71-77, 2004.

Article 1 states, on page 869, right column, lines 16 to 20, "The enhancing effects of SCE on humoral immune responses may affect local mucosal immune responses which may correspond with the onset of specific immunity to E. tenella infection." SCE is an abbreviation for sugar cane extract as set forth on page 865, right column, lines 8 to 9 of Article 1, and obtained using the method of the present applicant as set forth on page 866, left column, lines 6 to 10 of Article 1.

Article 2 describes, on page 989, right column, lines 9 to 12, "The improving effects of SCE on immuno-suppression in CPA-treated chickens may be due to the recovery of the bursa-dependent lymphoid system and stimulation of immunocomponent B cells for morphological reconstitution of the bursa of CPA-treated chickens."

Article 3 mentions, on page 77, left column, lines 1 to 4, "the efficacy of SCE as one of immunostimulants which can protect and/or recover from X-ray radiation induced immunosuppression in chickens..."

In addition, Article 4, Osamu Nakamura et al., "Suppression of Salmonella Enteritidis Excretion in Chicks Fed on a Sugar Cane Extract", Proceedings of the 132nd Research Seminar of the Japanese Society of Veterinary Science, page 199, PS-6109, September 7, 2001, is also enclosed. Inventor Takeo Mizutani is one of the authors of Article 4. Article 4 teaches that the sugar cane-derived extract has an immunostimulating effect against *Salmonella Enteritidis*.

As seen from the specification and the four articles discussed above, it is clear that the sugar cane-derived extract of the invention works against infections by all types of bacteria, virus and fungi through control of immunological function. Favorable consideration and withdrawal of the rejection is requested.

Claims 153-204 have been rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Examiner alleges that claims 153, 168 and 183, and their dependents, are rendered vague and indefinite by reciting the phrase, "providing a

remedial effect for a disease" because the phrase is not adequately defined by the claim language or specification. This rejection is respectfully traversed and reconsideration is requested for the reasons, which follow.

Symptoms caused by a bacterial, fungal or viral infection are various and dependent on each kind of bacteria, virus and fungi. However, the symptoms caused by a specific bacterium, fungus or virus, are well-known from, for instance, books or dictionaries (for example, P. J. Quinn et al., "Clinical Veterinary Microbiology", Wolfe publishing, 1994; Patrick R. Murray et al., "Manual of Clinical Microbiology", ASM press, 1995.) For example, well-known symptoms caused by an infection of human influenza virus are fever (usually high), headache, extreme tiredness, dry cough, sore throat, runny or stuffy nose, muscle aches, nausea, vomiting, and diarrhea.

Thus, the phrase, "providing a remedial effect for a disease" clearly communicates to a skilled person the provision of a remedy for these well-known symptoms. Therefore, the term, "providing a remedial effect for a disease" is sufficiently clear to meet the requirements of 35 U.S.C. 112. Favorable consideration and withdrawal of the rejection is requested.

The Rejection Under 35 U.S.C. §102

Claims 153-154, 168-169 and 183 have been rejected under 35 U.S.C. §102(b) as being anticipated by WO 98/02041 (Bermudez et al.) (hereinafter referred to as "Bermudez"). This rejection, at least insofar as it applies to claims 153-154, 168-169 and 183, as amended, is respectfully traversed and reconsideration is requested for the reasons that follow.

Bermudez discloses a method for preparing compositions useful in anti-adhesive therapies, comprising two heating steps as seen on page 3, lines 1 to 10 and page 5, lines 12 to 27 of Bermudez. Heating step 1 is to heat the filtered liquid extract at a temperature of from about 60°C to about 70°C for a time period of about 30 to about 60 minutes. Heating step 2 is to heat the liquid extract obtained in heating step 1 at a temperature of from about 130°C to about 165°C for about 24 hours. Bermudez also discloses that it is important that the extract reach a temperature of at least 150°C (i.e. the boiling point) as seen on page 5, lines 26 to 27 of Bermudez. The resulting product, Bercedin, contains 90 weight % of carbohydrates (mono-, oligo- and polysaccharides), as seen on page 6, lines 3 to 5 of Bermudez.

Bermudez also mentions, on page 2, lines 23 to 25 that, "it is believed that heat polymerization of one or more components of the sugar cane extract is required to develop the therapeutic activity of the composition."(emphasis added). Therefore, it is seen that one or more heat polymerized carbohydrates or polysaccharides are the active components in the Bercedin of Bermudez.

The sugar cane-derived extracts of the present invention do not undergo heating above a temperature of 130°C during the preparation process. It is well known that sugar cane juice, sugar cane-derived molasses and a liquid extract of sugar cane with water, methanol, ethanol and a mixture thereof (hereinafter referred to as "raw materials") are prepared at a temperature of less than 120°C, as will be explained below in item (A). Then, steps of column chromatography (B) and concentration of eluents (C) are performed at a temperature of 100°C or less, as described below, to prepare the present extract.

(A) Raw materials

Sugar cane juice and sugar cane-derived molasses are prepared at a temperature of less than 120°C. This is because it is necessary to prevent degradation of sucrose in a process for recovering sucrose. If the raw material is heated to a temperature above 130°C, as set forth in Bermudez, sucrose will be degraded and polymerized.

In a first aspect, the sugar cane juice includes mill juice obtained by milling sugar cane, extracted juice obtained by extracting sugar cane with water, clarified juice obtained by treating with lime in a sugar mill, and concentrated juice as seen on page 14, lines 13 to 16 of the specification. In a process for obtaining mill juice, water or warm water is used in milling. If the temperature is higher than 40°C, the milling machine is inoperable due to dissolution of waxy components from sugar cane in water.

The temperature of the hot water used in a process for obtaining extracted juice is usually from 70 to 80°C. See Reference 5, George P. Meade, "Spencer-Meade Cane Sugar Handbook NINTH EDITION" – a manual for cane sugar manufacturers and their chemists, John, Wiley & Sons, Inc., 1963, page 60, lines 26 to 27, "Steam is also added directly to the diffusing boots and the temperature of the mill-expressed juices other than the first are about 170°F". 170°F equates to 77°C.

Clarified juice is obtained by clarifying mill juice or extracted juice. In a clarifying process, mill juice or extracted juice is heated to a temperature of from 90 to 115°C (see Reference 5, page 89, second line from the bottom, to page 91, line 2). According to the general opinion in the year 1963, the temperature was just above the boiling point of water (103°C)(see Reference 5, page 91, line 5).

Concentrated juice is obtained by concentrating the mill juice, the extracted juice or the clarified juice. In the concentration process, multiple-effect evaporators are usually used for heating, and the liquid temperature is 54 to 120°C, more generally 96 to 106°C. When sugar liquid is concentrated to a specific sugar concentration in one evaporator, the concentrated sugar liquid is transferred to the next evaporator. The temperature and pressure decrease downwardly. The temperature and pressure are such that the sugar liquid boils to evaporate water. The sugar liquid in the last evaporator has a concentration of Bx 70, whose boiling point is 100 + 5.5°C (see Reference 5, page 149, line 7). No more elevation of the liquid temperature occurs in the multi-effect evaporators.

In another process where sugar liquid is heated under pressure, the maximum temperature is less than 120°C. If a higher temperature is applied, the ratio of sugar recovery will deteriorate due to heat decomposition.

In a second aspect, the sugar cane-derived molasses includes those from a sugar mill; and those from a sugar refinery, as seen on page 14, lines 23 to 29 of the specification. In a sugar mill, sucrose seed crystals are added to the concentrated juice (see above) to obtain a mixture of precipitated sucrose crystals and a mother liquid, which is then centrifuged to separate the sucrose crystals, leaving the molasses. As mentioned above, no temperature higher than 120°C is applied up to the concentration process for obtaining the concentrated juice.

The temperature applied in the crystallization step will now be discussed. Although the sugar liquid becomes supersaturated to cause boiling point-elevation, the temperature of boiling sugar remains at 50 to 105°C in a reduced pressure pan. See Reference 5, page 191, line 14, "with 83 purity and a strike temperature of 150°F". 150°F equates to 66°C.

In a sugar refinery, sucrose crystals obtained from a sugar mill are dissolved in water, which is then clarified. The juice thus obtained is concentrated using multiple-effect evaporators. No heating over 100°C is applied in any of the clarification, concentration and

crystallization steps where the purity of sucrose is rather high so that decomposition of sugar may be avoided.

Also, use may be made of a residue of molasses that is deprived of saccharides, such as an isolated liquor obtained in alcoholic fermentation of such molasses, as seen on page 14, lines 29 to 31 of the specification. Alcohol has a low boiling point, compared to that of water, and therefore the distillation temperature in a still is less than 100°C.

In a third aspect, the liquid extract of sugar cane may be obtained by heating a mixture of sugar cane or bagasse, which is obtained by milling in water or warm water, as mentioned above, with water, methanol, ethanol or a mixture thereof. The boiling points of methanol, ethanol and water and a mixture thereof are: 64.65°C, 78.32°C, 100°C and less than 100°C, respectively. Thus, the raw material has not been heated above 120°C.

(B) Column chromatography

The raw material is subjected to column chromatography below a temperature of 97°C as discussed below.

(a) The raw material obtained in one of the manners explained above is passed through a column packed with a synthetic adsorbent as a fixed carrier at a temperature of 60 to 97°C. Substances adsorbed on the synthetic adsorbent are eluted with a solvent at a temperature of 20 to 40°C. See page 18, lines 13 to 18 of the specification.

(b) The raw material obtained in one of the manners explained above is passed through a ion chromatographic column packed with a synthetic adsorbents as a fixed carrier at a temperature of 40 to 70°C. Substances adsorbed on the synthetic adsorbent are eluted with a solvent at a temperature of 40 to 70°C. See page 19, line 27 to page 20, line 4 of the specification.

(C) Concentration of the eluents

The eluents thus obtained are condensed under vacuum in a concentrator below 105°C and optionally freeze-dried below 0°C.

Thus, the sugar cane-derived extract of the invention has never experienced a temperature above 130°C, as is required by the Bermudez reference.

The active ingredients of Bercedin in Bermudez are carbohydrates (mono-, oligo- and polysaccharides). On the other hand, an active ingredient of the invention, as claimed in the amended claims, is one or more non-saccharides. Therefore, the active ingredient of the invention is different than that of the Bercedin of Bermudez. Favorable consideration and withdrawal of the rejection under 35 U.S.C. §102(b) is requested.

The Rejection Under 35 USC §103

Claims 153-204 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Bermudez, Kawai, Saska, Agar, Brewer and Kearny. This rejection is respectfully traversed and reconsideration is requested for the reasons that follow.

As demonstrated above, the active ingredients of Bercedin in Bermudez are carbohydrates (mono-, oligo- and polysaccharides). The present claims have been limited to require one or more non-saccharide active ingredients. Thus, this element of all of the claims is not taught or suggested by Bermudez.

Kawai, Saska, Agar, Brewer and Kearney all relate to various aspects of the sugar cane extract manufacturing process, but none of these references provides any teaching or suggestion to modify the composition of the primary reference, Bermudez, to employ a non-saccharide active ingredient. In fact, such a modification would never be obvious in view of Bermudez since, as discussed above, Bermudez contains a clear teaching that the active ingredients of the Bermudez compositions must be saccharides. Thus, the present invention is not realized from the combination of these references.

Moreover, Bermudez differs from many sugar cane extraction processes in that Bermudez requires temperatures above 130°C, whereas, in many sugar cane extraction processes, such high temperatures are avoided in order to minimize degradation of the desired product sucrose. As a result, it is not obvious to combine the method of Bermudez with the secondary references to Kawai, Saska, Agar, Brewer and Kearney since Bermudez desires polymerization of carbohydrates at temperatures above 130°C, whereas the other references would avoid such high temperatures and polymerization in order to preserve the highest possible concentration of sucrose, the desired end product of these references.

Finally, in response to the Examiner's comment in the office action on page 9, lines 5 to 7 of the Office Action, wherein the Examiner alleges that the amended claims read on eating

sugar because the claimed extracts are obtained by the same methods practiced by the references to obtain sugar, the applicant disagrees since, as demonstrated above, the method of Bermudez is clearly different than the methods of the present invention. Moreover, the claimed method clearly does not read on eating sugar because the claimed method expressly requires the administration of "a sugar cane-derived extract" and not "sugar" as the Examiner suggests.

Favorable consideration, entry of the amendment and issuance of a Notice of Allowance are solicited. Should the Examiner have any questions she is encouraged to call the Applicant's representative listed below.

Respectfully submitted,

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Article 1

FULL PAPER *Immunology*

Protective Effects of Sugar Cane Extracts (SCE) on *Eimeria tenella* Infection in Chickens

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ABSTRACT. The effects of oral administration of sugar cane extracts (SCE) on *Eimeria tenella* oocysts infection in chickens were studied with 2 different experiments. In Experiment 1, 3-week-old inbred chickens (MHC; H.B15) were inoculated into the crop with SCE (500 mg/kg/day) for 1 day or 3 consecutive days, and then challenged with *E. tenella* sporulated oocysts (2×10^4 cells/chicken). In Experiment 2, 1-week-old chickens were orally administered SCE at the same dose for 3 consecutive days, and then initially infected with *E. tenella* sporulated oocysts (2×10^3 cells/chicken). At 2 and 3 weeks of age, these chickens were immunized intravenously with the mixed antigens of sheep red blood cells (SRBC) and *Brucella abortus* (BA). At 4 weeks of age, chickens were challenged with *E. tenella* sporulated oocysts (1×10^5/chicken). Challenged chickens with *E. tenella* oocysts showed markedly decreased body weight gain/day, severe hemorrhage and great number of shedding oocysts in feces and high lesion scores. Oral administration of SCE and initial infection with oocysts (2×10^3/chicken) resulted in a remarkable improvement in body weight gain/day, hemorrhage, the number of shedding oocysts and lesion score, compare to other infected groups. In addition, SCE-inoculated chickens with the initial infection showed a significant increase in antibody responses against SRBC and BA and also improvement in decreased relative proportions of Bu-1a$^+$ and CD4 cells in cecal tonsil lymphocytes of *E. tenella*-challenged chickens. Cecal tissues of chickens administered SCE and initially infected with *E. tenella* oocysts showed lower numbers of schizonts, gametocytes and oocysts than those of infected control chickens. These results suggest that SCE have immunostimulating and protective effects against *E. tenella* infection in chickens.

KEY WORDS: *Eimeria tenella*, protective effect, sugar cane extract.

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Chicken coccidiosis is caused by obligate intestinal protozoan parasites belonging to several different species of *Eimeria*. *Eimeria tenella* (*E. tenella*) primarily invades and resides in the lining of the cecum of exposed chickens [1, 14, 27–30]. Infective sporozoits enter the cecal mucosa by penetrating villus epithelial cells, resulting in extensive destruction of the cecal epithelium, hemorrhagic feces, reduction in body weight gain, and decrease in feed efficiency and eventual mortality which lead to serious economic consequences. Thus far, chemoprophylaxis and anticoccidial feed additives have controlled the disease but have been complicated by the emergence of drug resistance [4, 12, 26].

To prevent the emergence of drug resistance, new drugs have been developed and administered on a rotational basis with existing drugs. However, this has resulted in the increased cost of poultry products. Furthermore, drug or antibiotic residue in the poultry products is potentially annoyance to consumer. Therefore, the regulation of anticoccidial drugs should be strengthened gradually. It is generally agreed that protective immunity does not block sporozoites from penetrating the intestinal epithelium, but it is not clear to what extent immune response to *E. tenella* sporozoites in the gut lumen can block penetration. Chickens orally administered attenuated vaccine or infected with a small number of *E. tenella* oocysts were shown to be resistant against challenge infection with the sublethal number of the same parasite.

Immunomodulators are a highly expanding field of studies to compete and/or control infectious diseases. Sugar cane extracts (SCE), one of native immunostimulants, have been reported to enhance immune responses, immune functions and growth in chickens [5, 6]. In immune chickens only a few sporozoites reach the cecal epithelium during reinfection and those are unable to develop further [22, 23, 27]. The purpose of the present study is to explore the feasibility of immunological protection of chickens against *E. tenella* infection by oral administration of SCE and initial infection with *E. tenella* oocysts.

MATERIALS AND METHODS

Animals: Inbred chickens (MHC; H.B15), maintained at the National Institute of Animal Health (NIAH), Tsukuba, Japan, were reared coccidia-free with free access to food and water.

Feed: Experimental feed without antibiotics and anticoccidial food additives was obtained from food manufacture

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company (Sugiji Co., Ltd., Handa, Japan).

Parasite and infection: *Eimeria tenella* (*E. tenella*), the NIAH strain, was used. The strain was originally isolated from naturally infected birds and maintained at the NIAH, Tsukuba, Japan.

SCE administration: Original materials of SCE including cane juice produced from sugar cane (*Saccharum officinarum* L.) in the raw sugar manufacturing process were subjected to the preparation of SCE (Shin Mitsui Sugar Co., Ltd., Japan) as described earlier [5, 6]. The original concentration of SCE (100 mg/m*l*) was prepared in phosphate buffered saline (PBS). SCE was inoculated with a pipette (Komagome pipet, Iwaki Co., Tokyo, Japan) into the crop of chickens at the dose of 500 mg/kg/day for 1 day or 3 consecutive days.

Experiment 1: This experiment was carried out to evaluate the effect of SCE on the incidence of coccidiosis in chickens. Three-week-old inbred chickens were divided into 4 groups, 1) uninfected control group (referred to as control), 2) chickens were inoculated into the crop with *E. tenella* oocysts (2×10^4 cells/chicken) (referred to as challenge), 3) chickens orally administered a single dose of SCE (500 mg/kg) and then challenged with the same dose of *E. tenella* oocysts (referred to as SCE (1) + challenge) and 4) chickens orally administered SCE for 3 consecutive days and then challenged with *E. tenella* oocysts (referred to as SCE (3) + challenge).

The body weight was measured at days 0 and 7 after challenge. Mortality was recorded and body weight gain per day was calculated. Hemorrhagic feces were observed from day 5 to day 7 post challenge. The total amount of feces was collected from all experimental groups from day 5 to day 7 after challenge for oocyst shedding determination [19]. The oocysts were diluted and counted microscopically in a plankton counter chamber. Total oocyst number was calculated as oocyst count × dilution factor × counting chamber volume × fecal sample volume. On day 7 post challenge all chickens were killed and scored for gross cecal lesions on a scale of 0 to 4 according to the method of Conway [3].

Experiment 2: This experiment was performed to evaluate the effects of SCE on immune responses and infection against *E. tenella* in chickens. First, 1-week-old chickens were divided into three groups of control, initial infection and SCE (3) + initial chickens, which were referred in Experiment 1. Each group consisted of 12 chickens. All chickens were immunized intravenously with the mixed antigens of sheep red blood cells (SRBC) and *Brucella abortus* (BA) at 2 and 3 weeks of age. Subsequently, at 4 weeks of age after evaluation of antibody responses to SRBC and BA, half chickens in the above three groups were challenged orally with *E. tenella* sporulated oocysts (1×10^5/chicken), consisting the following six groups; 1) saline-administered uninfected control chickens (control), 2) chickens challenged with *E. tenella* sporulated oocysts (1×10^5/chicken) (challenge), 3) chickens challenged with *E. tenella* sporulated oocysts (2×10^3/chicken) (initial infection), 4) chickens initially infected and then challenged with *E. tenella* oocysts (initial + challenge), 5) chickens administered SCE for 3 consecutive days and then initially infected with *E. tenella* oocysts (SCE (3) + initial), and 6) chickens administered SCE for 3 consecutive days, initially infected and then challenged with *E. tenella* oocysts (SCE (3) + initial + challenge). Each group consisted of six chickens. Gain in body weight/day (g/day), clinical signs, oocyst shedding and lesion scores were evaluated in all chickens after challenge at 4 weeks of age.

Immunization and determination of antibody titers: Each chicken was immunized intravenously with 0.1 m*l* of mixed antigens containing SRBC (5×10^8 cells) and BA (1×10^9 cells) [8] at 2 and 3 weeks of age. Agglutinin titers against SRBC and BA were evaluated in sera taken at 7 days after each immunization, as described previously [8]. The sera were also treated with 0.2 M 2-mercaptoethanol (ME) to evaluate 2-ME resistant agglutinin titers.

Isolation of intestinal leukocytes: Chicken intestinal leukocytes (IL) were prepared according to the technique of Choi *et al.* [2]. Briefly, the intestine between the duodenal loop and the region immediately prior to the Meckel's diverticulum was excised, cut longitudinally, and washed 3 times in PBS supplemented with 2% fetal bovine serum (FBS). Intestinal tissue of each chicken was treated separately, cut into small pieces, and incubated for 10 min in the same medium supplemented by 10 mM dithiothreitol to eliminate the intestinal mucous membrane. The supernatant was discarded and the small pieces of the intestine were incubated for 20 min at 37°C in PBS containing 1 mM EDTA. Cells in the supernatant were washed and gently pressed through a stainless steel mesh to remove most epithelial cells, dead cells and cellular clusters. Cells were further purified by 60% percoll density gradient centrifugation at 3,000 rpm for 20 min at 24°C to remove red cells. Cell viability was over 95% as determined by trypan blue exclusion. The cells were finally suspended to a concentration of 1×10^6 cells/m*l* in 2% FBS-PBS.

Relative proportions of cells positive for marker antigens: The relative proportions (RP) of cells positive for marker antigens in IL were evaluated the following procedures described by Erf *et al.* [7]. Briefly, 100 μl of chicken leukocytes (1×10^6 cells/m*l*) and 100 μl of 2% FBS-PBS were mixed in each well of a 96-well plate and centrifuged at 2,000 rpm for 1 min at 4°C. After 2 times washing, the sedimented cells were supplemented with 100 μl of mouse-anti-chicken monoclonal antibody specific for Bu-1a, CD4, CD8, TCR1, TCR2 and TCR3 markers, generous gifts of Dr. Olli Vainio, University of Turku, Finland, and then incubated on ice for 30 min. After incubation the cells were washed 2 times with 2% FBS-PBS and supplemented with 100 μl of FITC-conjugated anti-mouse IgG antibody (Zymed, CA, U.S.A.) diluted 1:400 in PBS. The cells were incubated on ice for 30 min, and then washed 2 times with 2% FBS-PBS. The RP of Bu-1a$^+$, CD4$^+$, CD8$^+$, TCR1$^+$, TCR2$^+$ and TCR3$^+$ cells in IL were evaluated using a flow cytometer (XL, Beckman Coulter Corp, U.S.A.).

Histopathology: On day 7 post challenge cecal tissue

samples were excised, fixed with neutral buffered formalin (10%), and then embedded in paraffin. Approximately 4 μm thick cross sections were excised and stained with hematoxylin and eosin (HE) for histopathological examination [19].

Efficacy of SCE: Efficacy of SCE was evaluated on the basis of mortality, gain in body weight/day, degree of hemorrhagic feces, oocyst count/chicken, lesion scores, immune responses and RP of Bu-1a$^+$ and CD4$^+$ in IL. The mortality was estimated from the number of dead chickens in each infected group. The body weight gain per day was determined from challenge to 1 week post challenge. The extent of hemorrhagic fecal score was assigned corresponding to the degree of hemorrhages in the feces [29]. Oocyst shedding was investigated from day 6 to day 7 post challenge with *E. tenella* and the lesion score of each group was investigated on day 7 post challenge. The localization of schizonts, gametocytes and oocysts in the cecum was microscopically investigated.

Statistical analysis: The Student's *t* test was used for statistical significance determination. P values of less than 0.05 were considered to be statistically significant. All data were expressed as mean ± standard error (SE).

RESULTS

Clinical signs and lesion scores in Experiment 1: Chickens challenged with *E. tenella* (2×10^4) showed severe clinical signs such as anorexia, depression, severe and continuous hemorrhages in feces and hemorrhagic feces around the cloaca. The surviving chickens showed retarded growth with decreased body weight gain/day as shown in Table 1. Yellowish-white sausage-like structures in the feces excreted from the cecum could be found. In contrast, chickens orally administered SCE and challenged with oocysts showed no mortality, indicating a significant improvement in the body weight gain/day, milder hemorrhages and less number of oocysts shed in feces and lower lesion scores, when compared to the challenged groups (Table 1).

Immune responses: The results concerning immune responses against SRBC and BA in Experiment 2 are summarized in Table 2. SCE-administration and additional initial infection with *E. tenella* oocysts (2×10^3) resulted in an increase in antibody responses against SRBC and BA in both first and second responses, as compared with those of uninfected control group and initially infected group. In addition, the enhancing effects of SCE were shown in both

Table 1. Effect of oral administration of SCE on body gain, fecal oocyst shedding and lesion score in *E. tenella* infected chickens (Experiment 1)

Group	Number of total chickens	Number of dead chickens	Gain in body weight (g/day)[a]	Hemorrhagic feces[b]	Oocyst shed/chicken ($\times 10^6$)[c]		Lesion score[d]
					Day 6	Day 7	
Control	10	0	10.7 ± 0.1	–	0	0	0
Challenge	12	0	7.6 ± 1.2*	++	58	27	+4.0
SCE (1) + challenge	7	0	8.6 ± 1.1	+	23	16	+3.8
SCE (3) + challenge	12	0	8.9 ± 0.9**	+	16	12	+3.3

a) Mean ± SE.
b) +: Transient hemorrhage; ++: continuous hemorrhage from day 5 to day 7 post infection.
c) Values represent mean oocyst numbers in pooled feces of each group.
d) Values represent mean lesion scores of each group. * P<0.01, compared to control group, and ** P<0.05, compared to challenge group.

Table 2. Antibody responses to SRBC and BA in chickens orally administered SCE and initially infected with *E. tenella* oocysts (Experiment 2)

Group	SCE	Initial infection with oocysts (2×10^3)	Immune responses[a]							
			SRBC				BA			
			First response		Second response		First response		Second response	
			Number of responders	Titer	Number of responders	Titer	Number of responders	Titer	Number of responders	Titer
Control	–	–	12/12	8.0 ± 0.7	12/12	10.3 ± 0.6	1/12	0.4 ± 0.4	12/12	10.8 ± 1.0
			12/12	(6.0 ± 0.3)[b]	12/12	(6.7 ± 0.5)	0/12	(0)	0/12	(0)
Initial infection	–	+	12/12	7.4 ± 0.7	12/12	10.2 ± 0.9	1/12	0.2 ± 0.2	12/12	10.7 ± 0.7
			12/12	(5.8 ± 0.2)	12/12	(7.9 ± 0.4)	0/12	(0)	0/12	(0)
SCE (3) + initial	+	+	12/12	12.2 ± 1.0**	12/12	13.6 ± 0.7*	4/12	2.6 ± 1.0*	12/12	13.8 ± 1.0
			12/12	(8.3 ± 0.5)**	12/12	(11.1 ± 0.6)**	0/12	(0)	0/12	(0)

a) Mean ± SE of $\log_2$ of the reciprocal antibody titer.
b) The parenthesis show 2-ME resistant titers.
* P<0.05; ** P<0.01, compared to control and initially infected groups, respectively.

Table 3. Mortality rate, gain in body weight, fecal oocyst shedding and lesion score in chickens orally administered SCE and infected with *E. tenella* oocysts (Experiment 2)

Group	SEC	*E. tenella* Initial (2×10^3)	*E. tenella* Challenge (1×10^5)	Number of total chickens	Number of dead chickens	Gain in body weight (g/day)[a]	Hemorrhagic feces[b]	Oocysts shed /chicken ($\times 10^6$)[c]	Lesion score[d]
Control	–	–	–	6	0	10.6 ± 0.8	–	0	0
Challenge	–	–	+	6	1	8.5 ± 1.4	++	42.0	+4.0
Initial infection	–	+	–	6	0	11.7 ± 1.7	–	0	0
Initial + challenge	–	+	+	6	0	11.0 ± 0.7	+	40.0	+3.0
SCE (3) + initial	+	+	–	6	0	15.9 ± 0.4*	–	0	0
SCE (3) + initial + challenge	+	+	+	6	0	13.9 ± 0.9*	+	3.3	+2.0

a) Mean ± standard error.
b) +: Transient hemorrhage; ++: continuous hemorrhage from day 5 to day 7 post infection.
c) Values represent mean oocyst numbers in pooled feces of each group.
d) Values represent mean lesion scores of each group.
* $P<0.01$, compared to control, challenge and initial + challene groups.

Table 4. Relative proportions (RP) of cells positive for marker antigens in intestinal leukocytes (IL) of chickens orally administered SCE and infected with *E. tenella* oocysts (Experiment 2)

Group	SCE	*E. tenella* Initial (2×10^3)	*E. tenella* Challenge (1×10^5)	RP (%) of surface marker antigen positive cells[a] BU-1a$^+$	CD4$^+$	CD8$^+$	TCR1$^+$	TCR2$^+$	TCR3$^+$
Control	–	–	–	11.8	10.2	34.1	12.5	16.5	12.5
Challenge	–	–	+	5.7	3.7	50.7	23.5	19.5	18.5
Initial infection	–	+	–	14.4	21.0	35.9	13.4	26.5	10.5
Initial + challenge	–	+	+	11.0	12.8	55.0	18.0	32.0	12.7
SCE (3) initial	+	+	–	24.5	10.5	35.4	28.8	15.5	9.3
SCE (3) + initial + challenge	+	+	+	15.8	7.5	49.5	36.9	19.7	18.5

a) RP was examined on day 8 after challenge. Values represent the mean PR of 3 chickens in each group.

numbers and titers of responding chickens producing antibodies to BA in the first and second responses.

Gain in body weight, oocyst shedding and lesion scores in Experiment 2: All chickens except the three groups of control, initial infection and SCE (3) + initial in Experiment 2 were challenged with *E. tenella* oocysts (1×10^5). Challenged chickens showed retarded growth as determined by sharply decreased body weight gain/day (8.5 ± 1.4 g/day) as shown in Table 3. Severe and continuous hemorrhages in feces and hemorrhagic feces around the cloaca were also observed in those chickens. In addition, challenged group and initially infected + challenged group shed a large number of oocysts in the feces/chicken on days 6 to 7 (42×10^6 and 40×10^6, respectively) after challenge infections with typical gross lesions including mucosal edema, severe hemorrhagic intestinal inflammation, gray-white to milky yellow sausage-like structures composed of oocysts, necrotic cells, red blood cells and mucous filling the intestinal lumen and necrosis of the cecum and lower parts of small intestine (lesion score, +4 and +3, respectively). On the other hand, chickens administered SCE and initially infected with oocysts showed improved body weight gain/day (13.9 ± 0.9 g/day), milder hemorrhages and less number of oocysts (3.3×10^6) shed in the feces and mild intestinal inflammation with lower lesion score (+2) after challenge, when compared with the described two groups (Table 3).

RP of surface marker antigens: The results concerning the mean RP in IL from 3 chickens in each group are summarized in Table 4. Chickens challenged with *E. tenella* oocysts showed decreased RP of Bu-1a$^+$ and CD4$^+$ cells (3.7%), as compared to control (11.8% and 10.2%, respectively). On other hand, the RP of Bu-1a$^+$ cells and CD4$^+$ cells in SCE (3) + initial + challenge group were 15.8% and 7.8%, respectively, suggesting that oral administration of SCE just before initial infection with oocysts improved these decreased RP in IL of *E. tenella*-challenged chickens. Furthermore an apparent increase in the RP of TCR1$^+$ cells was shown in chicken groups administered SCE, SCE (3) + initial and SCE (3) + initial + challenge, when compared to that of chickens not administered SCE.

Histopathology: As showed in Fig. 1, a lot of schizonts were observed in the cecal mucosa of challenged chickens (Fig. 1A) and initially infected + challenged chickens (Fig. 1B). On the other hand, very few schizonts were observed in cecal sections from SCE (3) + initial + challenge chickens (Fig. 1C).

DISCUSSION

The goal of the experiments in the present study was to determine the effects of oral administration of SCE on main variables associated with pathology caused by *E. tenella*

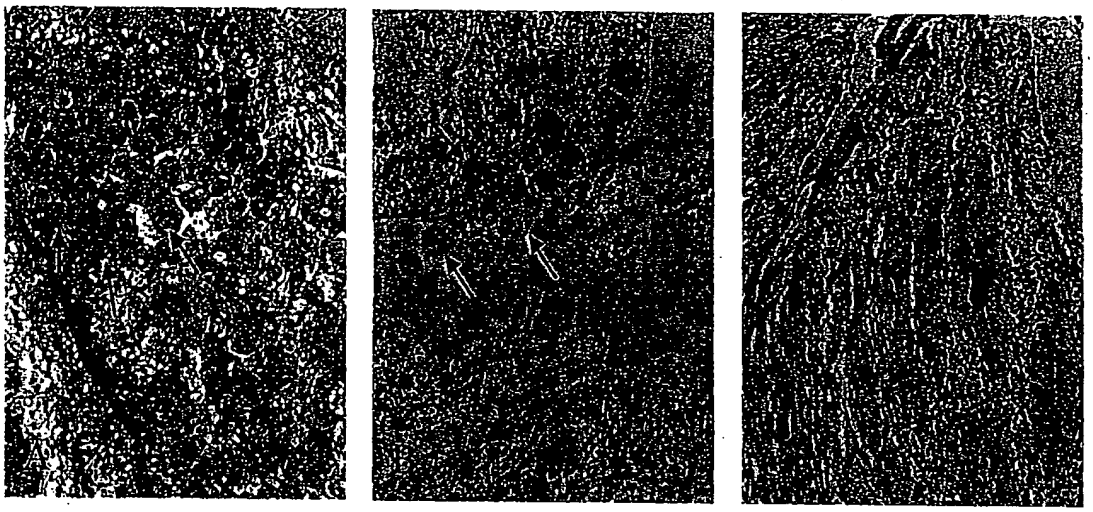

Fig. 1. Histology of the ceca of 5-week-old chickens challenged with *E. tenella* sporulated oocysts (1×10^5) (HE stain, × 100). A) A lot of schizonts (→) in the cecal mucosa of challenged chickens, B) many schizonts (→) in the cecal mucosa of initially infected + challenged chickens and C) no schizonts in the cecal mucosa of SCE + initially infected + challenged chickens.

infection in chickens including mortality, body weight gain, the degree of hemorrhage in feces, oocyst shedding, lesion score and immune responses. The body weight gain/day significantly decreased after challenge with *E. tenella* oocysts. Reduced body weight gain is a major contributor to production of loss that accompanies coccidial infection in young chickens because inflammatory immune responses divert energy from growth which may affect the weight gain [13]. Oral administration of SCE significantly improved body weight gain per day in initially infected chickens and initially infected + challenged chickens. These results confirmed our previous results concerning the growth-promoting effects of SCE in chickens [5, 6]. The improving effects of SCE on body weight gain and decreasing the mortality in chickens after *E. tenella* oocyst infection may be associated with its protective effects. Youn and Noh [29] reported that administration of herb extracts improved the survival rate and body weight gain in chickens infected with *E. tenella*. However, Allen [1] reported that daily oral administration of L-arginine (500 mg/kg) did not increase the body weight gain but significantly reduced oocyst shed from *E. tenella*-infected chickens than infected control group. The body weight gain/day, hemorrhages and oocyst shedding in feces and lesion scores were investigated during 1 week after challenge with *E. tenella* oocysts. Hemorrhages in feces of almost all experimental groups, except the uninfected control group, were seen during 5–7 days after infection with *E. tenella* oocyst. But the extent of hemorrhage in feces of chickens administered SCE and initially infected with oocysts was milder than that of other infected groups. The oocyst output in SCE-administered + initially infected + challenged chickens (3.3×10^6/chicken) was lower than that of challenged group and initially infected + challenged group (42 and 40×10^6/chicken, respectively). The lesion scores in the SCE + initially infected + challenged group (+2) improved better than those of challenged group and initially infected + challenged group (+4 and +3, respectively). These results indicated a protective effect of SCE on *E. tenella* infection.

The antibody responses against SRBC and BA significantly increased in both first and second responses in chickens orally administered SCE and initially infected with oocysts, when compared with those of uninfected control and initially infected chickens. These results also confirmed our previous findings that oral administration of SCE significantly increased the antibody responses against SRBC, BA and *Salmonella enteritidis* (SE) and also maintained higher antibody titers than control chickens [5], suggesting the stimulatory effects of SCE on antibody production. The enhancing effects of SCE on humoral immune responses may affect local mucosal immune responses which may correspond with the onset of specific immunity to *E. tenella* infection. These results are in agreement with those of Li *et al.* [15, 16] reported the activation of classical complement pathway by a polysaccharide from sugar cane extracts and its interaction with immunoglobulins. Parmentier *et al.* [20] reported immune responses and resistance to *Eimeria acervulina* of chickens divergently selected for antibody response to SRBC.

Flow cytometric analysis of cecal tonsil lymphocytes showed decreased relative proportions of Bu-1a$^+$ and CD4$^+$ cells in IL of challenged chickens. Oral administration of SCE with oocysts of *E. tenella* improved these decreased proportions in IL of challenged chickens with *E. tenella* oocysts, indicating the stimulatory effects of SCE on the local mucosal immunity. It is generally believed that CD4$^+$

cells are important to develop help during antibody responses, but $CD4^+$ cells also provide help during induction of cytotoxic responses [17, 25, 28]. Using the murine model system for protozoan infection, the importance of $CD4^+$ cells has been reported in the control of infection with *E. tenella* [24, 25] and *Toxoplasma gondii* [10, 11]. Stimulation of antibody responses to SRBC and BA in chickens orally administered SCE and initially infected with oocysts and improved relative proportions of Bu-$1a^+$ and $CD4^+$ cells in IL suggested that administration of SCE with a low dose of oocysts played a role in the development of protective immunity against oocyst-reinfection by interacting with lymphocytes. The importance of lymphocytes in immune responses to coccidia has been reported in chickens [23]. The spleen cells and peripheral blood lymphocytes from immune chickens are capable of transferring the resistance against the infection to naive recipients. Furthermore, the treatment of chickens with immunosuppressive agents enhanced the severity of coccidiosis. Isobe and Lillehoj [9] reported that dexamethasone-treated chickens showed reduced T-cell proliferation, reduced interferon production and increased susceptibility to *Eimeria* infection. It was reported that cane sugar factors induced *in vivo* protective responses against *Pseudomonas aeruginosa* and *Proteus mirabilis* [21]. Youn and Noh [29] reported anticoccidial effects of herbal extracts on *E. tenella* infection. In the present study oral administration of SCE with *E. tenella* oocysts was found to show more effective improvement after challenge than challenged group and initially infected + challenged group on the basis of survival rate, gain in body weight, hemorrhages and oocyst shedding in feces and lesion scores.

Histopathological examination revealed that chickens received SCE with initial infection showed lower numbers of schizonts, gametocytes and oocysts in the cecum after challenge than any other infected groups (even if initially infected with *E. tenella* oocysts). The lack of parasite development in cecal tissues, enhanced immune responses and improved relative proportions of Bu-$1a^+$ and $CD4^+$ cells in IL of chickens orally administered SCE and initially infected with oocysts indicated that these birds had mounted protective immune responses which may prevent invasion and development of sporozoites in the cecal tissue after challenge. It has been reported that avian humoral immunity in the intestinal tract is mediated via secretion of antibody by plasma cells located within the gut lamina propria into the intestinal lumen [1, 18]. Vervelde *et al.* [28] reported the major role of intestinal leukocytes in protective immunity following *E. tenella* infection. The mechanism by which oral administration of SCE results in reduction of pathological lesions in *E. tenella*-infected chickens remains open. Further basic studies including the interaction between SCE and intestinal immune cells are needed.

Taken together, these results suggest that inoculation of SCE with *E. tenella* oocysts into the crop induces protective immunity against *E. tenella* infection in chickens. The protective effects of SCE-administration on local mucosal as well as systemic immune responses may inhibit the invasion and/or natural development of the parasites.

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Article 2

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Preventive and therapeutic effects of sugar cane extract on cyclophosphamide-induced immunosuppression in chickens

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Preventive and therapeutic effects of sugar cane extract on cyclophosphamide-induced immunosuppression in chickens

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Abstract

Effects of oral administration of sugar cane extract (SCE) on immunosuppression in chickens treated with cyclophosphamide (CPA) were evaluated. Three-week-old inbred chickens were inoculated into the crop with SCE (500 mg/kg/day) for three consecutive days before or after injection of CPA 12 or 20 mg/chicken. At the last day of SCE or CPA treatment, all chickens were immunized intravenously with sheep red blood cells (SRBC) and *Brucella abortus* (BA). Chickens administered SCE showed a significant increase in body weight, gain in body weight/day, relative weight of the bursa of Fabricius and antibody responses to SRBC and BA than untreated control chickens. Chickens injected with CPA alone showed significantly decreased body weight, gain in body weight/day, relative weight of the bursa and antibody responses to SRBC and BA, showing immunosuppression in the bursa-dependent immune system. All chickens administered SCE before or after the treatment with CPA showed significantly higher values in body weight, gain in body weight/day, relative bursal weight and antibody responses to both antigens, when compared to chickens treated with CPA alone. In histological examination, chickens administered SCE showed a typical bursa with well constituted follicles, although chickens treated with CPA alone showed a severely atrophied bursa with rudimentary follicles and enormous proliferation of interfollicular connective tissue. Chickens treated with SCE and CPA showed a well-reconstituted bursa with almost normal structure. These results suggest that SCE has functionally and morphologically reconstituting effects on the bursa-dependent immune system in immunosuppressed chickens induced by injection of CPA.

Keywords: Chickens; Immunosuppression; Cyclophosphamide; Sugar cane extract

1. Introduction

Immunosuppression caused by many factors including infection and stress is a common biological phenomenon in animals. Despite the development of

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potent new antibiotics, the frequency of opportunistic infections [1,2], primarily attributed to stress-associated immunosuppression, has increased in economically important domestic food animals, causing great economic losses. Moreover, the high incidence of drug-resistant bacteria from the frequent and continuous use of antibiotics poses big problems facing the poultry industry. A possible application of immunostimulants of a native type such as chicken egg white derivatives to clinical practice of companion animals with immunosuppression-associated diseases has been reported [3,4].

We have been reported that sugar cane extract (SCE) enhances the growth rate, antibody responses to sheep red blood cells (SRBC), *Brucella abortus* (BA) and a commercially available *Salmonella* Enteritidis vaccine and delayed type hypersensitivity responses to human γ globulin in chickens [5]. Furthermore, SCE has been found to have radioprotective effect [6] on intestinal damage in X-ray irradiated chickens and protective effects against *Eimeria tenella* oocyst infection in chickens [7,8].

Cyclophosphamide (CPA) is an immunosuppressive agent widely used in organ transplantation and the treatment of various autoimmune disorders [9]. It has been reported that injection of CPA to newly hatched chickens primarily induced selective B cell damage resulting in irreversible humoral immunosuppression [10–14]. Animals with deficient humoral immunity resulted in increased susceptibility to infection [15]. It is necessary to find a useful immunopotentiator for enhancing the immunotherapeutic value of CPA treatment and improving productivity of animal farms.

Therefore, the purpose of the present study is to examine the effects of oral administration of SCE on functional and histological reconstitution of the bursa-dependent immune system in chickens intramuscularly injected with CPA.

2. Materials and methods

2.1. Animals

Inbred chickens of line V (MHC; H.B15), maintained at the National Institute of Animal Health (NIAH), Tsukuba, Japan, were used in the present study. The present study including the treatment of chickens is followed according to the Guidelines for Animal Experiments of NIAH, Japan.

2.2. SCE

SCE was kindly provided by Shin Mitsui Sugar, Japan. Original materials including cane juice produced from sugar cane (*Saccharum officinarum* L.) in the raw sugar manufacturing process were subjected to the preparation of SCE. Dried SCE finally prepared by synthetic adsorbent chromatography and cation exchange column chromatography consisted of crude protein (16.9%), crude fat (0.5%), ash (36.1%) and nitrogen-free extracts (46.5%) [6]. The original concentration (100 mg/ml) of SCE was prepared in 0.1 M phosphate buffered saline (PBS), and inoculated into the crop of chickens at the dose of 500 mg/kg/day for three consecutive days with a round bottom type KomagomeTM pipette (Iwaki Glass, Tokyo, Japan). Chickens, orally administered physiological saline instead of SCE in the same manner, were subjected to a control group.

2.3. CPA

Four milligrams (Experiment 1) and 5 mg (Experiment 2) of CPA (EndoxanTM, Shinogi, Osaka, Japan) was injected into the femoral muscle of a chicken for three and four consecutive days, respectively [13].

2.4. Experiment protocol

In Experiment 1, 3-week-old chickens were divided into six groups: (1) saline-administered control chickens (referred to as control), (2) chickens received CPA alone (4 mg/chick/day for three consecutive days) (CPA), (3) chickens administered SCE alone for three consecutive days (SCE), (4) chickens administered SCE for three consecutive days and then injected with CPA for three consecutive days (SCE + CPA), (5) chickens received CPA for three consecutive days and then administered SCE for three consecutive days (CPA + SCE), and (6) chickens administered SCE and injected with CPA at the same time (CPA = SCE).

In this Experiment 2, 3-week-old chickens were treated with CPA at 5 mg/chick/day for four consec-

utive days to induce more severe immunosuppression than that in Experiment 1, and then divided into six groups with the same reference described above.

2.5. Body weights and relative weights of the bursa of Fabricius and spleen

Chickens were weighed individually before SCE and CPA administration at the age of 3 weeks and weighed again 3 weeks later. The body weight gain/day in each chicken group was calculated. At 6 weeks of age, birds were killed with ether. The bursa and spleen were excised, and then collected in sterile PBS. The relative organ weights were evaluated and expressed as mg/100 g body weight [16].

2.6. Immunization and evaluation of antibody titers

At 28 and 35 days of age, on the last day of SCE administration or CPA treatment, each chicken was immunized intravenously with 0.1 ml of mixed antigens containing sheep red blood cells (SRBC: 5×10^8 cells) and heat-inactivated *B. abortus* (BA: 1×10^9 cells). Agglutinin titers against SRBC and BA were determined in heat-inactivated sera taken 7 days after each immunization, as procedures described by Hirota et al. [17]. The sera were also treated with 0.2 M 2-mercaptoethanol (2-ME) to determine 2-ME resistant agglutinin titers. Agglutinin titers were expressed as the mean $\log_2$ of the reciprocal of the highest dilution giving 50% agglutination.

2.7. Microscopic examination of the bursa

The bursa and spleen tissues were fixed with neutral buffered formalin (10%), and embedded in paraffin. Approximately 4-μm-thick tissue sections were stained with hematoxylin and eosin (HE) for histopathological examination.

2.8. Statistical analysis

The Student's *t* test and Kruskal–Wallis/Mann–Whiteney *U*-test were used for statistical significance determination. *P* values of less than 0.05 were considered to be statistically significant. All data were expressed as the mean ± standard error of the mean (S.E.M.).

3. Results

3.1. Body weights and the relative weights of the bursa and spleen

The results concerning body weight, body weight gain/day and relative weight of the bursa and spleen in Experiments 1 and 2 are summarized in Tables 1

Table 1
The effects of SCE on body weight, the gain in body weight and the relative weight of bursa and spleen in 6-week-old chickens injected intramuscularly with CPA (4 mg/day) for three consecutive days at 3 weeks of age (Experiment 1)[a]

Group	Number	Body weight		Gain in body weight (g)	Bursal weight (mg/100 g body weight)	Spleen weight (mg/100 g body weight)
		At 3-week-old (g)	At 6-week-old (g)			
Control	5	79.0 ± 6.5	242.8 ± 5.4	7.6 ± 0.3	446 ± 26	293 ± 52
CPA	6	93.5 ± 10.8	233.0 ± 3.8	6.5 ± 0.5	273 ± 14**	280 ± 34
SCE	6	93.5 ± 10.5	280.0 ± 10.0*	8.6 ± 0.4[a,†]	520 ± 20[a,††]	346 ± 9
SCE + CPA	6	80.0 ± 6.8	249.8 ± 5.9	8.0 ± 0.3[†]	390 ± 45[†]	343 ± 40
CPA + SCE	6	80.2 ± 7.0	238.0 ± 7.4	7.5 ± 0.3	300 ± 36	317 ± 40
CAP = SCE	6	80.5 ± 10.0	243.0 ± 10.0	7.6 ± 0.4	450 ± 57[†]	347 ± 27

[a] Three-week-old chickens were injected intramuscularly with CPA (4 mg/chicken/day) for three consecutive days (referred to CPA) or orally administered SCE (500 mg/kg/day) for three consecutive days (SCE). Before or after the treatment with CPA, chickens were orally administered SCE (SCE + CPA and CPA + SCE, respectively). The group referred to CAP = SCE was chickens that received cotreatment with CPA and SCE at the same day in the same manner. All data except for body weight show values (mean ± S.E.M.) measured at the age of 6 weeks.

*$P < 0.05$, when compared to control.

**$P < 0.01$, when compared to control.

†$P < 0.05$, when compared to CPA.

††$P < 0.01$, when compared to CPA.

Table 2
The effects of SCE on body weight, the gain in body weight and the relative weight of bursa and spleen in 6-week-old chickens injected intramuscularly with CPA (5 mg/day) for four consecutive days at 3 weeks of age (Experiment 2)[a]

Group	Number	Body weight		Gain in body weight (g)	Bursal weight (mg/100 g body weight)	Spleen weight (mg/100 g body weight)
		At 3-week-old (g)	At 6-week-old (g)			
Control	6	99.6 ± 15.7	245.5 ± 29.1	7.0 ± 0.9	466.0 ± 06	244.1 ± 31.4
CPA	7	85.0 ± 10.4	225.2 ± 10.7	6.5 ± 0.7	96.3 ± 11.0**	205.3 ± 5.9
SCE	7	96.5 ± 10.3	255.5 ± 1.9*	7.6 ± 0.4	502.5 ± 12.2*,††	297.3 ± 15.9††
SCE+CPA	7	98.3 ± 15.9	246.0 ± 14.5	7.0 ± 0.7	167.7 ± 49.4†	250.5 ± 50.7
CPA+SCE	7	94.5 ± 9.8	237.0 ± 3.8	6.8 ± 0.4	146.8 ± 33.0	275.6 ± 43.7
CAP=SCE	7	94.5 ± 18.0	245.0 ± 25.1	7.0 ± 0.8	169.0 ± 22.9††	235.5 ± 5.9†

[a] Three-week-old chickens were injected intramuscularly with CPA (5 mg/chicken/day) for four consecutive days (referred to CPA) or orally administered SCE (500 mg/kg/day) for three consecutive days (SCE). Before or after the treatment with CPA, chickens were orally administered SCE (SCE+CPA and CPA+SCE, respectively). The group referred to CAP=SCE was chickens received measured at the age of 6 weeks.
*$P<0.05$, when compared to control.
**$P<0.01$, when compared to control.
†$P<0.05$, when compared to CPA.
††$P<0.01$, when compared to CPA.

and 2, respectively. Chickens orally administered SCE showed higher body weight, body weight gain/day and relative weight of the bursa than untreated control chickens, when evaluated at 6 weeks of age. Chickens treated with CPA at the total doses of 12 or 20 mg showed a significant decrease in body weight, body weight gain/day and relative weight of the bursa and spleen, when compared to control chickens ($P<0.05$). Each value in chickens treated with 20 mg of CPA was lower than that in

Table 3
The effects of SCE on antibody responses to SRBC and BA in 6-week-old chickens injected intramuscularly with CPA (4 mg/day) for three consecutive days at 3 weeks of age (Experiment 1)[a]

Group	SRBC				BA			
	First response		Second response		First response		Second response	
	Responders/total number	Titer	Responders/total number	Titer	Titer Responders/total number	Titer	Responders/total number	Titer
Control	5/5	9.0 ± 0.4	5/5	9.4 ± 0.2	5/5	6.6 ± 1.1	5/5	11.2 ± 0.5
	(4/5	2.4 ± 0.8)[b]	(5/5	5.0 ± 0.3)	(0/5	0)	(2/5	1.4 ± 0.9)
CPA	6/6	7.0 ± 0.6*	6/6	8.1 ± 0.3*	3/6	3.0 ± 1.1*	6/6	12.3 ± 0.2
	(3/6	1.0 ± 0.4)*	(6/6	4.2 ± 0.4)	(0/6	0)	(0/6	0)
SCE	6/6	11.1 ± 0.4**,††	6/6	11.7 ± 0.2**,††	6/6	8.7 ± 0.3††	6/6	15.7 ± 0.2††
	(6/6	4.0 ± 0.2)††	(6/6	8.2 ± 0.3)**,††	(0/6	0)	(5/6	3.2 ± 0.7)*,†
SCE+CPA	6/6	9.3 ± 0.0††	6/6	9.8 ± 0.2††	6/6	7.7 ± 0.8††	6/6	13.0 ± 0.2*
	(6/6	2.7 ± 0.2)††	(6/6	5.8 ± 0.3)††	(0/6	0)	(4/6	2.2 ± 0.8)*
CPA+SCE	6/6	7.5 ± 0.0	6/6	10.3 ± 0.2††	6/6	5.0 ± 0.7††	6/6	10.5 ± 0.3
	(5/6	2.3 ± 0.0)	(6/6	6.3 ± 0.4)*,††	(0/6	0)	(0/6	0)
CAP=SCE	6/6	9.8 ± 0.0*	6/6	8.4 ± 0.3	6/6	3.6 ± 0.6	6/6	9.2 ± 0.2
	(5/6	2.3 ± 0.2)	(6/6	4.7 ± 0.3)	(0/6	0)	(1/6	0.3 ± 0.3)

[a] Three-week-old chickens of all groups treated with CPA (total dose, 12 mg) or SCE (500 mg/kg/day) were immunized intravenously with SRBC and BA at 4 and 5 weeks of age. Agglutinin to both antigens in sera taken 7 days after each immunization was evaluated. All values represent mean ± S.E.M. of $\log_2$ titers.
[b] The parentheses show 2-ME resistant titers.
*$P<0.05$, when compared to control.
**$P<0.01$, when compared to control.
†$P<0.05$, when compared to CPA.
††$P<0.01$, when compared to CPA.

chickens treated with 12 mg of CPA, suggesting their more sever situation. In addition, all chicken groups of SCE+CPA, CPA+SCE and CPA=SCE were higher in any parameters of body weight, gain in body weight/day and relative weight of the bursa and spleen than chickens treated with CPA alone.

3.2. Immune responses to SRBC and BA

Immune responses to SRBC and BA in Experiments 1 and 2 are shown in Tables 3 and 4. The antibody titers significantly increased in the first and second responses to both antigens in chickens orally administered SCE, when compared to those of control chickens ($P<0.05$). Negligible or low antibody responses to both antigens were shown in CPA-treated chickens. The magnitude of the antibody responses in chickens treated with 20 mg of CPA was lower than that of chickens treated with 12 mg of CPA, suggesting a dose-dependent immunosuppression of B cell-dependent immune system. Chickens in the SCE+CPA groups revealed significantly higher antibody responses against both antigens than chickens injected with CPA alone. In addition, cotreatment of SCE and CPA resulted in an increase in both numbers and titers of responding chickens producing 2-ME resistant antibodies to SRBC and BA in the first and second responses (Tables 2 and 3).

3.3. Histology of the bursa

The bursa of chickens treated with CPA alone revealed only rudimentary follicles with very few, if any, lymphoid cells, no demarcation between cortex and medulla and enormous proliferation of interfollicular connective tissue (Figs. 1B and 2B). Chickens administered SCE showed the typical bursa with well-constituted follicles (Figs. 1C and 2C). The bursal follicles of chicken groups injected with CPA for three or four consecutive days and then administered SCE were well reconstituted by lymphoid cells and larger (Figs. 1E and 2E), when compared to those of groups treated with CPA alone. The bursa of SCE+CPA

Table 4
The effects of SCE on antibody responses to SRBC and BA in 6-week-old chickens injected intramuscularly with CPA (5 mg/day) for four consecutive days at 3 weeks of age (Experiment 2)[a]

Group	SRBC				BA			
	First response		Second response		First response		Second response	
	Responders/ total number	Titer	Responders/ total number	Titer	Titer Responders/ total number	Titer	Responders/ total number	Titer
Control	6/6	10.5 ± 0.6	6/6	10.6 ± 0.6	6/6	6.0 ± 2.0	6/6	8.6 ± 0.6
	(6/6	3.0 ± 0.5)[b]	(6/6	5.3 ± 0.3)*	(0/6	0)	(2/6	1.3 ± 0.6)
CPA	7/7	5.8 ± 0.3**	7/7	8.6 ± 0.8*	0/7	0**	4/7	5.0 ± 1.4
	(0/7	0)††	(4/7	2.8 ± 0.7)	(0/7	0)	(0/7	0)
SCE	7/7	12.5 ± 0.2**,††	7/7	13.0 ± 0.0**,††	7/7	8.5 ± 0.2††	7/7	8.8 ± 0.4†
	(7/7	3.3 ± 0.2)††	(7/7	6.8 ± 0.2)**,††	(0/7	0)	(7/7	3.3 ± 0.6)
SCE+CPA	7/7	10.7 ± 0.8††	7/7	12.3 ± 0.3*,†	7/7	3.7 ± 1.2††	7/7	8.6 ± 0.8
	(7/7	2.0 ± 0.0)	(7/7	4.3 ± 0.3)††	(0/7	0)	(7/7	2.0 ± 0.0)
CPA+SCE	7/7	9.8 ± 0.5††	7/7	11.0 ± 1.0	7/7	3.8 ± 1.1††	7/7	8.6 ± 1.4
	(4/7	1.8 ± 1.2)	(7/7	3.7 ± 0.6)	(0/7	0)	(7/7	2.0 ± 0.5)
CAP=SCE	7/7	9.8 ± 0.9††	7/7	9.8 ± 1.4	4/7	0.5 ± 0.2	7/7	5.0 ± 1.4
	(7/7	2.3 ± 0.4††	(7/7	3.3 ± 0.6)	(0/7	0)	(2/7	0.3 ± 0.2)

[a] Three-week-old chickens of all groups treated with CPA (total dose, 20 mg) or SCE (500 mg/kg/day) were immunized intravenously with SRBC and BA at 4 and 5 weeks of age. Agglutinin responses to both antigens in sera taken 7 days after each immunization were evaluated. All values represent mean ± S.E.M. of $\log_2$ titers.

[b] The parentheses show 2-ME resistant titers.

*$P<0.05$, when compared to control.

**$P<0.01$, when compared to control.

†$P<0.05$, when compared to CPA.

††$P<0.01$, when compared to CPA.

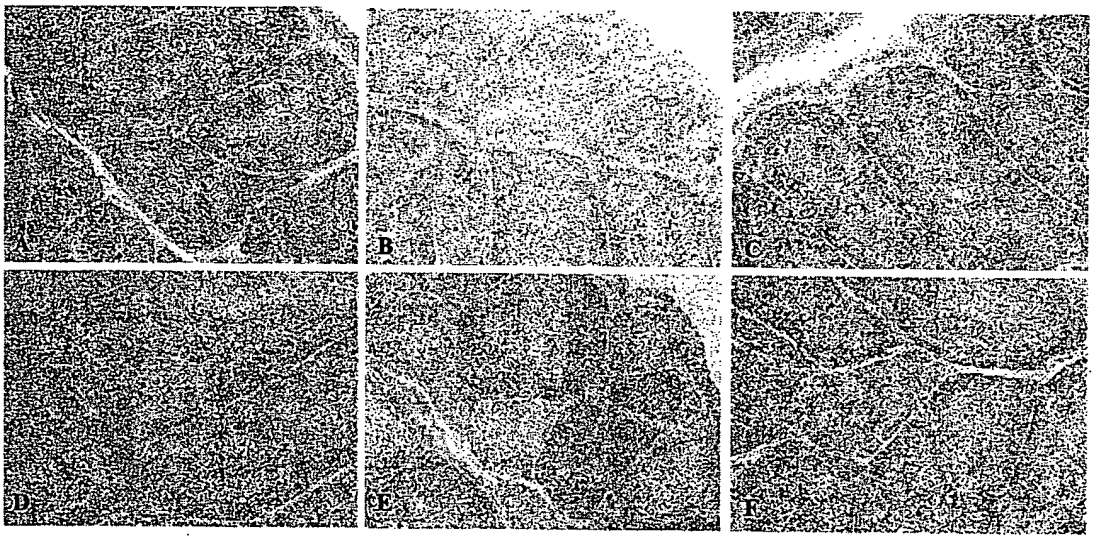

Fig. 1. Reconstituting effects of SCE on the bursal structure in 6-week-old chickens treated with CPA (12 mg) at 3 weeks of age. (A) Control, (B) CPA, (C) SCE, (D) SCE + CPA, (E) CPA + SCE and (F) CPA = SCE (HE-stain, magnification × 100).

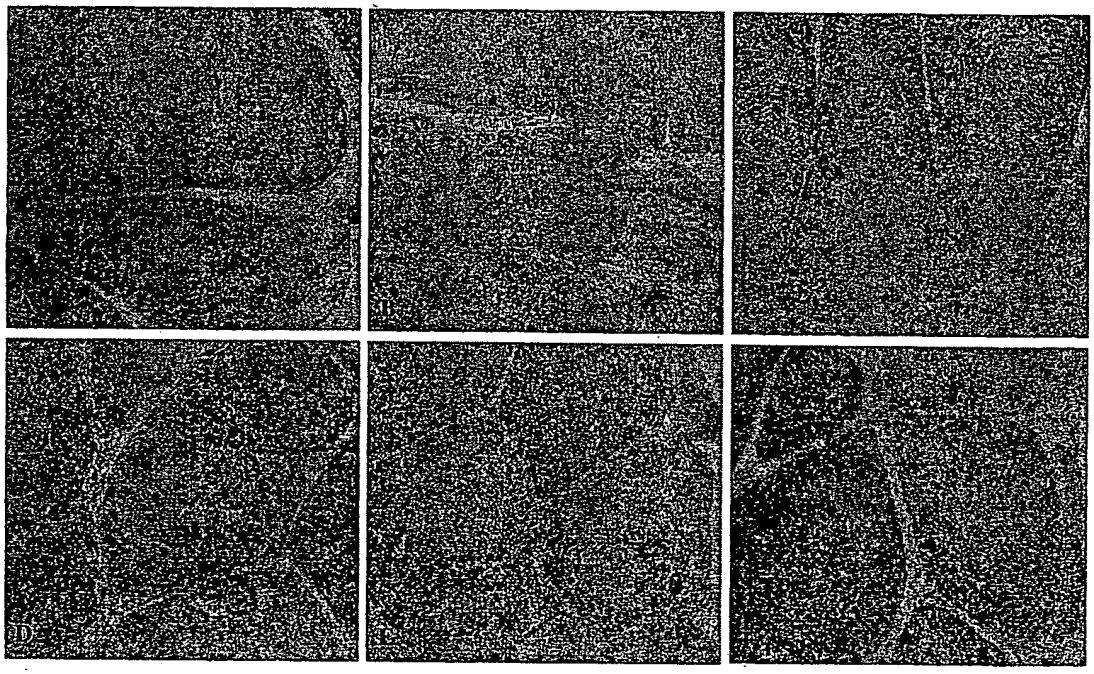

Fig. 2. Reconstituting effects of SCE on the bursal structure in 6-week-old chickens treated with CPA (20 mg) at 3 weeks of age. (A) Control, (B) CPA, (C) SCE, (D) SCE + CPA, (E) CPA + SCE and (F) CPA = SCE (HE-stain, magnification × 100).

(Figs. 1D and 2D) and CPA=SCE groups (Figs. 1F and 2F) also showed a histologically well-reconstituted bursa with improved follicular structure and decreased interfollicular fibrosis, when compared with chickens treated with CPA alone.

4. Discussion

The results of the present study can be summarized as follows. (1) Severe immunosuppression was demonstrated in CPA-treated chickens as shown in a decreased body weight, gain in body weight/day, bursal weights, immune responses to SRBC and BA and histologically degenerative alterations of the bursa associated with a depletion of lymphoid cells and atrophy. (2) Cotreatment with SCE and CPA resulted in functional and morphological reconstitution of the bursa-dependent lymphoid system. The total doses (12 and 20 mg/chicken) of CPA used were determined to cause chemical bursectomy in newly hatched chickens [12,13,16] and marked reduction in body weight and bursal weight at 3 weeks of age [11]. It has been reported that the treatment of chickens with CPA resulted in a significant decrease in body weight and bursal weights [12,13,16,18]. B lymphocytes severely decreased or disappeared in lymphoid organs such as the bursa, spleen and cecal tonsil of CPA-treated chickens [10]. Oral administration of SCE improved the body weight, gain in body weight and the relative weight of the bursa in CPA-treated chickens, suggesting that SCE has reconstituting effects on the lymphoid system. Chicken peripheral blood polymorphonuclear cells cultured with SCE showed an increase in their phagocytosis, suggesting the direct effects of SCE on leukocytes. An increase in the relative proportion of CD4+cells in peripheral blood lymphocytes and plaque-forming cell responses of splenocytes of chickens orally administered SCE (data not shown), and improvement in antibody responses to SRBC and BA and relative weights of the bursa in CPA-treated chickens suggested indirect effects of SCE on the immune system.

Reynolds and Maraqa [18] also reported the immunosuppressive effects induced by injection of CPA resulted in a severe lymphoid depletion of B cells in peripheral blood lymphocytes of CPA-treated birds, retarded body weight and no production of antibodies specific for Newcastle disease virus in chickens. In the present study, the decreased immune responses to SRBC and BA in CPA-treated chickens were improved by coadministration of SCE. The enhancing effect of SCE was revealed, in particular when evaluated in both numbers and titers of responding chickens producing 2-ME resistant antibodies to SRBC and BA. The improving effects of SCE on immunosuppression in CPA-treated chickens may be due to the recovery of the bursa-dependent lymphoid system and stimulation of immunocompetent B cells for morphological reconstitution of the bursa of CPA-treated chickens.

CPA-treated chickens showed a morphologically degenerative alteration including the atrophied bursa with undeveloped follicles. It has been reported that CPA-treated neonatal chickens have a virtual depletion of lymphoid cells in the bursa of Fabricius, decreased thymic cortical cells and absence of lymphoid cells and plasma cells in the spleen [19]. Chickens injected with CPA and irradiated with X-ray completely suppressed antibody responses, immunoglobulin production and formation of bursal follicles and splenic germinal centers [13]. Transplantation of bursal cells into chickens immunologically suppressed by CPA-treatment restored the suppressed humoral immune system in both function and structure. The improvement of humoral immune responses and reconstitution of bursal follicles in CPA-treated chickens were obtained by coadministration of SCE. The mechanisms by which SCE are involved in the improvement of CPA-induced immunosuppression remain open. One possible mechanism is that administration of SCE into the crop of chickens induces compensatory proliferation or regeneration of lymphocytic progenitors and immunocompetent B cells suppressed by CPA.

Taken together, SCE is suggested to have improving and alleviating effects on CPA-associated immunosuppression.

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Erratum

Erratum to "Preventive and therapeutic effects of sugar cane extract on cyclophosphamide-induced immunosuppression in chickens" [International Immunopharmacology 418 (2004) 983–990]

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The publisher regrets errors that appeared in Tables 1–4 and the Corresponding author details of the above paper. The corrected items can be found below.

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Table 1
The effects of SCE on body weight, the gain in body weight and the relative weight of bursa and spleen in 6-week-old chickens injected intramuscularly with CPA (4 mg/day) for three consecutive days at 3 weeks of age (Experiment 1)[a]

Group	Number	Body weight		Gain in body weight (g)	Bursal weight (mg/100 g body weight)	Spleen weight (mg/100 g body weight)
		At 3-week-old (g)	At 6-week-old (g)			
Control	5	79.0±6.5	242.8±5.4	7.6±0.3	446±26	293±52
CPA	6	93.5±10.8	233.0±3.8	6.5±0.5	273±14**	280±34
SCE	6	93.5±10.5	280.0±10.0*	8.6±0.4[a,†]	520±20[a,††]	346±9
SCE+CPA	6	80.0±6.8	249.8±5.9	8.0±0.3[†]	390±45[†]	343±40
CPA+SCE	6	80.2±7.0	238.0±7.4	7.5±0.3	300±36	317±40
CPA=SCE	6	80.5±10.0	243.0±10.0	7.6±0.4	450±57[†]	347±27

[a] Three-week-old chickens were injected intramuscularly with CPA (4 mg/chicken/day) for three consecutive days (referred to CPA) or orally administered SCE (500 mg/kg/day) for three consecutive days (SCE). Before or after the treatment with CPA, chickens were orally administered SCE (SCE+CPA and CPA+SCE, respectively). The group referred to CPA=SCE was chickens that received cotreatment with CPA and SCE at the same day in the same manner. All data except for body weight show values (mean±S.E.M.) measured at the age of 6 weeks.

* $P<0.05$, when compared to control.

** $P<0.01$, when compared to control.

† $P<0.05$, when compared to CPA.

Table 2
The effects of SCE on body weight, the gain in body weight and the relative weight of bursa and spleen in 6-week-old chickens injected intramuscularly with CPA (5 mg/day) for four consecutive days at 3 weeks of age (Experiment 2)[a]

Group	Number	Body weight		Gain in body weight (g)	Bursal weight (mg/100 g body weight)	Spleen weight (mg/100 g body weight)
		At 3-week-old (g)	At 6-week-old (g)			
Control	6	99.6±15.7	245.5±29.1	7.0±0.9	466.0±06	244.1±31.4
CPA	7	85.0±10.4	225.2±10.7	6.5±0.7	96.3±11.0**	205.3±5.9
SCE	7	96.5±10.3	255.5±1.9*	7.6±0.4	502.5±12.2*,††	297.3±15.9††
SCE+CPA	7	98.3±15.9	246.0±14.5	7.0±0.7	167.7±49.4[†]	250.5±50.7
CPA+SCE	7	94.5±9.8	237.0±3.8	6.8±0.4	146.8±33.0	275.6±43.7
CPA=SCE	7	94.5±18.0	245.0±25.1	7.0±0.8	169.0±22.9[a,††]	235.5±5.9[†]

[a] Three-week-old chickens were injected intramuscularly with CPA (5 mg/chicken/day) for four consecutive days (referred to CPA) or orally administered SCE (500 mg/kg/day) for three consecutive days (SCE). Before or after the treatment with CPA, chickens were orally administered SCE (SCE+CPA and CPA+SCE, respectively). The group referred to CPA=SCE was chickens received measured at the age of 6 weeks.

* $P<0.05$, when compared to control.

** $P<0.01$, when compared to control.

† $P<0.05$, when compared to CPA.

†† $P<0.01$, when compared to CPA.

Table 3
The effects of SCE on antibody responses to SRBC and BA in 6-week-old chickens injected intramuscularly with CPA (4 mg/day) for three consecutive days at 3 weeks of age (Experiment 1)[a]

Group	SRBC				BA			
	First response		Second reponse		First response		Second response	
	Responders/ total number	Titer	Responders/ total number	Titer	Titer Responders/ total number	Titer	Responders/ total number	Titer
Control	5/5	9.0±0.4	5/5	9.4±0.2	5/5	6.6±1.1	5/5	11.2±0.5
	(4/5	2.4±0.8)[b]	(5/5	5.0±0.3)	(0/5	0)	(2/5	1.4±0.9)
CPA	6/6	7.0±0.6*	6/6	8.1±0.3*	3/6	3.0±1.1*	6/6	12.3±0.2
	(3/6	1.0±0.4)*	(6/6	4.2±0.4)	(0/6	0)	(0/6	0)
SCE	6/6	11.1±0.4**,††	6/6	11.7±0.2**,††	6/6	8.7±0.3††	6/6	15.7±0.2††
	(6/6	4.0±0.2)††	(6/6	8.2±0.3)**,††	(0/6	0)	(5/6	3.2±0.7)*,†
SCE+CPA	6/6	9.3±0.0††	6/6	9.8±0.2††	6/6	7.7±0.8††	6/6	13.0±0.2*
	(6/6	2.7±0.2)††	(6/6	5.8±0.3)††	(0/6	0)	(4/6	2.2±0.8)*
CPA+SCE	6/6	7.5±0.0	6/6	10.3±0.2††	6/6	0.0±0.7††	6/6	10.5±0.3
	(5/6	2.3±0.0)	(6/6	6.3±0.4)*,††	(0/6	0)	(0/6	0)
CPA=SCE	6/6	9.8±0.0*	6/6	8.4±0.3	6/6	5.6±0.6	6/6	9.2±0.2
	(5/6	2.3±0.2)	(6/6	4.7±0.3)	(0/6	0)	(1/6	0.3±0.3)

[a] Three-week-old chickens of all groups treated with CPA (total dose, 12 mg) or SCE (500 mg/kg/day) were immunized intravenously with SRBC and BA at 4 and 5 weeks of age. Agglutinin to both antigens in sera taken 7 days after each immunization was evaluated. All values represent mean±S.E.M. of $\log_2$ titers.

[b] The parentheses show 2-ME resistant titers.

* $P<0.05$, when compared to control.

** $P<0.01$, when compared to control.

† $P<0.05$, when compared to CPA.

†† $P<0.01$, when compared to CPA.

Table 4
The effects of SCE on antibody responses to SRBC and BA in 6-week-old chickens injected intramuscularly with CPA (5 mg/day) for four consecutive days at 3 weeks of age (Experiment 2)[a]

Group	SRBC				BA			
	First response		Second reponse		First response		Second response	
	Responders/ total number	Titer	Responders/ total number	Titer	Titer Responders/ total number	Titer	Responders/ total number	Titer
Control	6/6	10.5±0.6	6/6	10.6±0.6	6/6	6.0±2.0	6/6	8.6±0.6
	(6/6	3.0±0.5)[b]	(6/6	5.3±0.3)*	(0/6	0)	(2/6	1.3±0.6)
CPA	7/7	5.8±0.3**	7/7	8.6±0.8*	0/7	0**	4/7	5.0±1.4
	(0/7	0)[‡]	(4/7	2.8±0.7)	(0/7	0)	(0/7	0)
SCE	7/7	12.5±0.2*,††	7/7	13.0±0.0**,††	7/7	8.5±0.2††	7/7	8.8±0.4†
	(7/7	3.3±0.2)††	(7/7	6.8±0.2)**,††	(0/7	0)	(7/7	3.3±0.6)
SCE+CPA	7/7	10.7±0.8††	7/7	12.3±0.3*,†	7/7	3.7±1.2††	7/7	8.6±0.8
	(7/7	2.0±0.0)	(7/7	4.3±0.3)††	(0/7	0)	(7/7	2.0±0.0)
CPA+SCE	7/7	9.8±0.5††	7/7	11.0±1.0	7/7	3.8±1.1††	7/7	8.6±1.4
	(4/7	1.8±1.2)	(7/7	3.7±0.6)	(0/7	0)	(7/7	2.0±0.5)
CPA=SCE	7/7	9.8±0.9††	7/7	9.8±1.4	4/7	0.5±0.2	7/7	5.0±1.4
	(7/7	2.3±0.4)††	(7/7	3.3±0.6)	(0/7	0)	(2/7	0.3±0.2)

[a] Three-week-old chickens of all groups treated with CPA (total dose, 20 mg) or SCE (500 mg/kg/day) were immunized intravenously with SRBC and BA at 4 and 5 weeks of age. Agglutinin responses to both antigens in sera taken 7 days after each immunization were evaluated. All values represent mean±S.E.M. of $\log_2$ titers.

[b] The parentheses show 2-ME resistant titers.

* $P<0.05$, when compared to control.

** $P<0.01$, when compared to control.

† $P<0.05$, when compared to CPA.

†† $P<0.01$, when compared to CPA.

Article 3

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Immunostimulating effects of sugar cane extract on X-ray radiation induced immunosuppression in the chicken

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Abstract

The present study was undertaken to evaluate the effect of sugar cane (*Saccharum officinarum* L.) extract (SCE) on the immune system of X-ray immunosuppressed chickens. SCE (500 mg/kg/day) was administrated into the crop of 3-week-old chickens for three consecutive days before or after irradiation. The results indicated that administration of SCE before or after whole body X-ray irradiation enhanced both primary and secondary immune responses in chickens immunized with sheep red blood cells and *Brucella abortus* (BA) as well as cell-mediated immunity measured by delayed type hypersensitivity to human γ-globulin.

Keywords: Chicken; Immunostimulation; X-ray radiation; Sugar cane extract

1. Introduction

Immune dysfunction is one of the deleterious manifestations of radiation and many drugs, which render the animals more susceptible to opportunistic infections. Restoration of normal physiological milieu and generalized increase in resistance against infections in such immunocompromised hosts seems paramount. Natural origin extracts, which have immunopotentiating effects, are promising candidates in such circumstances. Many extracts have proved to enhance the immune system in normal and/or immunocompromised hosts. Mistletoe extract enhanced the cellular and humoral adjuvant activity in mice [1] and stimulated maturation of dendritic cells in vitro [2]. Datta et al. [3] reported that herbal protein, CI-1, isolated from the leaves of *Cajanus indicus* enhanced the humoral immune and delayed type hypersensitivity (DTH) responses in mice. Background and the humoral immune response to ovalbumin increased in unprimed and primed mice treated with aqueous extract of Epimedii Herba [4]. In addition, powdered

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extract from *Echinacea angustifolia* (Echinacea extract) had stimulated immunocompetence of horses [5]. Flavonoid glycosides from *Urtica dioica* L. showed enhanced chemotactic and intracellular killing activities of neutrophils in vitro [6]. Ginseng extract proved an adjuvant effect on the immune responses to *Staphylococcus aureus* in dairy cattle [7] and to porcine parvovirus and *Erysipelothrix rhusiopathiae* in pigs [8]. Moreover, some algal-based extracts showed similar stimulating activities. *Chlorella vulgaris* extract enhanced resistance of mice to infection with *Listeria monocytogenes* through augmentation of cytokine profiles [9]. Ergosan preparation, based on algal *Laminaria digitata* extract, had immunostimulating effects in rainbow trout [10].

Sugar cane extract (SCE), one of natural byproducts of sugar cane industry, has been proved to have immunostimulating and adjuvant effects in normal chickens [11,12] and a reconstituting effect on the immune system in cyclophosphamide-treated chickens [13]. Furthermore, the SCE showed anticoccidial properties in chickens [14]. Due to the reported range of immunostimulating activities of SCE, the present study has been undertaken to evaluate the effects of oral administration of SCE on immune functions in sublethally X-ray irradiated chickens.

2. Materials and methods

2.1. SCE administration

SCE was kindly provided by Shin Mitsui Sugar, Tokyo, Japan. Original materials including cane sugar juice produced from sugar cane (*Saccharum officinarum* L.) in the raw sugar manufacturing process were subjected to the preparation of SCE. Dried SCE finally prepared by synthetic adsorbent chromatography and cation exchange column chromatography consisted of crude protein (16.9%), crude fat (0.5%) ash (36.1%) and nitrogen-free extracts (46.5%). The original concentration (100 mg/ml) of SCE was prepared in 0.1 M phosphate-buffered saline (PBS) (Nissui pharmaceutical, Tokyo, Japan) and inoculated into the crop of chickens at the dose of 500 mg/kg/day for three consecutive days with a round bottom type Komagome™ pipette (Iwaki Glass, Tokyo, Japan). Chickens orally administered PBS instead of SCE in the same manner were subjected to a control group.

2.2. Experimental groups

Inbred chickens (MHC; H.B15), maintained at the National Institute of Animal Health (NIAH), Tsukuba, Japan, were used in this study. The present study including the treatment of chickens is followed to the Guidelines for Animal Experiments of NIAH Japan. Thirty-five, 3-week-old chickens of both sexes were matched for weight and divided into five groups: control group without any treatment (referred to as control), SCE administered group (SCE), X-ray irradiated group (600 R), SCE before irradiation (SCE + 600 R) and SCE after irradiation (600 R + SCE).

2.3. Irradiation

Whole body irradiation was achieved through 150-kV X-ray machine (MBR-1520R; Hitachi Medical, Tokyo, Japan) using 0.2 mm Cu and 0.5 mm Al as added filters, 15 mA as current and target distance 52 cm. The chickens were irradiated in Perspex, well-ventilated chamber centered on the rotating base of the machine. The dose rate was 100 rad/min at the total dose of 600 rad.

2.4. Immunization and determination of antibody titers

Each chicken was immunized intravenously with 0.1 ml of mixed antigens containing heat-inactivated *Brucella abortus* (BA; 1×10^9 cells) and sheep red blood cells (SRBC; 5×10^8 cells) [15] at 3 and 4 weeks of age. Agglutinin titers against SRBC and BA were determined in sera taken at 7 days after each immunization. The sera were also treated with 0.2 M 2-mercaptoetanol (ME) to evaluate 2-ME resistant agglutinin titers. Agglutinin titers were expressed as the mean $\log_2$ of the reciprocal of the highest dilution giving 50% agglutination.

2.5. Delayed type hypersensitivity (DTH) response

One week after the second immunization, each chicken was sensitized with 1 ml of human γ globulin (HγG; 400 μg/ml) emulsified with complete Freund's adjuvant. Two weeks later, chickens were challenged intradermally in the right wattle with 0.1 ml of saline solution of HγG (400 μg/ml) and the left wattle with 0.1 ml of saline [16]. The thickness of the wattles was measured with a Vernier caliper at 24, 48 and 72 h after challenge. The net increase in the thickness was expressed in millimeter.

2.6. Relative lymphoid organ weight

After measuring DTH responses at 7 weeks of age, the body weight of chickens in all experimental groups was determined, and then the birds were euthanized by ether. The thymus, spleen and bursa were excised free from adhering tissues and weighed individually. The relative lymphoid organ weights were calculated according to the following equation:

$$\text{Relative lymphoid organ weight} = \frac{\text{organ weight (g)}}{\text{body weight (g)}} \times 100.$$

2.7. Statistical analysis

Kruskal–Wallis/Mann–Whitney *U*-test was used for analyzing the data. $P<0.05$ was considered to be statistically significant. All data were expressed as mean ± standard error (S.E.).

3. Results

3.1. Agglutinin responses

The results concerning agglutinin titers against SRBC and BA are summarized in Table 1. Whole

Table 1
Effect of SCE on antibody responses to SRBC and BA in X-ray irradiated chickens

Group	Immune responses[a]							
	SRBC				BA			
	First response		Second response		First response		Second response	
	No. of responders	Titer	No. of responders	Titer	No. of responders	Titer	No. of responders	Titer
Control	7/7	10.5 ± 0.1	7/7	10.2 ± 0.1	7/7	4.7 ± 0.2	7/7	6.4 ± 0.1
	7/7	(5.4 ± 0.2)[b]	7/7	(4.6 ± 0.2)	1/7	(0.3 ± 0.1)	2/7	(0.6 ± 0.2)
SCE	7/7	12.2 ± 0.1*	7/7	11.4 ± 0.2*	7/7	6.4 ± 0.2	7/7	8.0 ± 0.2
	7/7	(5.1 ± 0.1)	7/7	(4.9 ± 0.4)	0/7	(0.0)	7/7	(1.9 ± 0.2)*
600 R	7/7	8.9 ± 0.1*	7/7	9.4 ± 0.1*	5/7	0.7 ± 0.1*	7/7	6.6 ± 0.1
	7/7	(3.6 ± 0.1)*	7/7	(3.7 ± 0.3)	0/7	(0.0)	0/7	(0.0)
SCE + 600 R	7/7	9.1 ± 0.1*	7/7	10.6 ± 0.2	7/7	4.1 ± 0.3**	7/7	7.9 ± 0.1*,**
	7/7	(2.4 ± 0.2)*	7/7	(6.1 ± 0.3)**	0/7	(0.0)	3/7	(0.7 ± 0.2)
600 R + SCE	7/7	9.1 ± 0.1*	7/7	10.4 ± 0.1**	7/7	5.6 ± 0.1**	7/7	8.1 ± 0.1*,**
	7/7	(2.4 ± 0.1)*,**	7/7	(6.1 ± 0.3)**	0/7	(0.0)	2/7	(0.4 ± 0.1)

Three-week-old chickens were administered SCE at a dose of 500 mg/kg/day for three consecutive days. After the last administration, the chickens were irradiated and immunized with 0.1 ml of SRBC and BA two times at 1-week interval. Agglutinin titers against SRBC and BA were evaluated in sera taken at 7 days after each immunization. The sera were also treated with 0.2 M 2-mercaptoetanol (ME) to evaluate 2-ME resistant agglutinin titers. Agglutinin titers were expressed as the mean $\log_2$ of the reciprocal of the highest dilution giving 50% agglutination.

[a] Mean ± S.E. of $\log_2$ of the reciprocal antibody titer.

[b] The parentheses show 2-ME resistant titers.

* $P<0.05$, when compared to control.

** $P<0.05$, when compared to irradiated group.

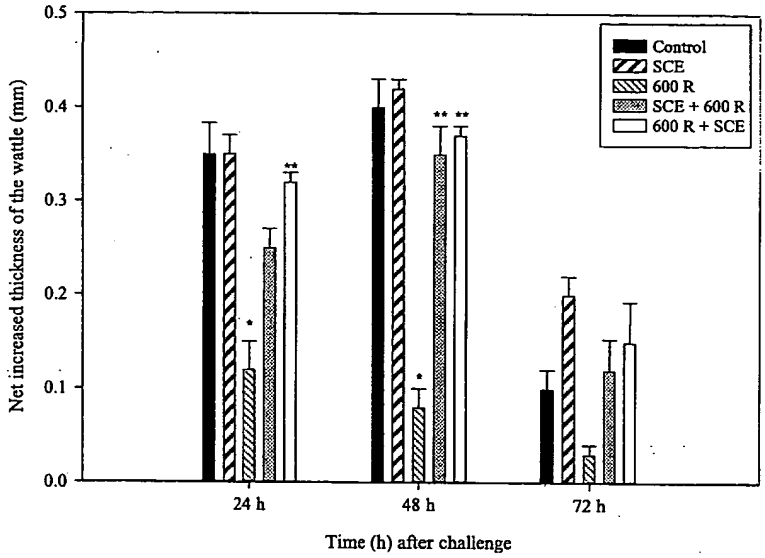

Fig. 1. Effects of SCE on DTH responses to human γ globulin in chickens. Three-week-old chickens were administered SCE at a dose of 500 mg/kg/day for three consecutive days. At 5 weeks of age, chickens were sensitized with 1 ml of HγG emulsified in complete Freund's adjuvant. Chickens were challenged by injection of 0.1 ml of saline solution of HγG in the right wattle, left wattle was injected with the same volume of saline. Net increase in the thickness of the wattle was determined at 24, 48 and 72 h after challenge. Values represent mean ± S.E. $*p<0.05$, when compared to control group and $**p<0.05$, when compared to 600 R group.

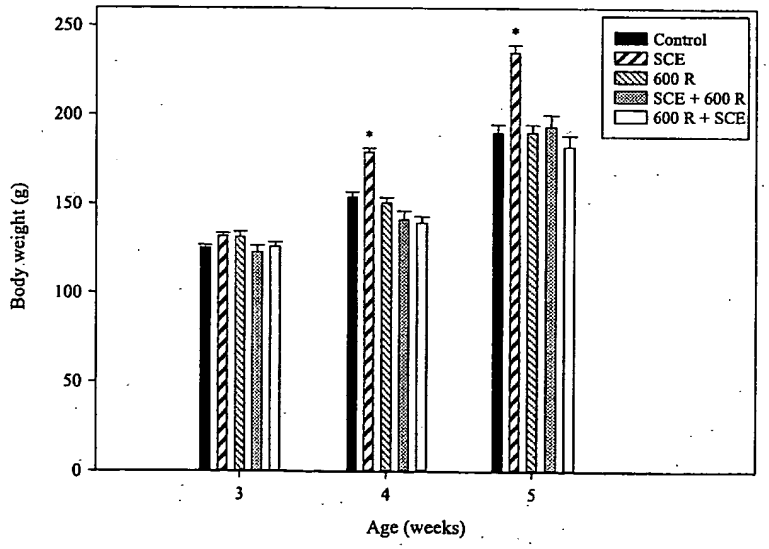

Fig. 2. Effects of SCE on the body weight of chickens. Three-week-old chickens were administered SCE at a dose of 500 mg/kg/day for three consecutive days. Body weight was measured at 3, 4, and 5 weeks of age. Values represent mean ± S.E. $*p<0.05$, when compared to control group.

body X-ray irradiation resulted in profound inhibition of the first immune response to BA and less pronounced decrease in the response to SRBC. SCE administration resulted in significantly enhanced total antibody titers in the first and second responses to both antigens. Although the anti-SRBC primary responses in SCE + 600 R and 600 R + SCE groups were higher than those of 600 R group, the magnitude of the agglutinin titers in both groups was less than that of untreated controls. Noteworthy, the response to BA was normalized in the groups of SCE + 600 R and 600 R + SCE and even showed a significant increase when compared to those of control and 600 R chickens in the second response. Interestingly, 2-ME resistant titers to SRBC in those chickens fluctuated between the apparent decrement and increment in the first and second responses, respectively.

3.2. DTH responses

As shown in Fig. 1, 600 rad X-ray whole body irradiation in chickens resulted in significantly suppressed DTH responses to HγG. SCE administration resulted in sustained responses of unirradiated chickens at 72 h after challenge, and normalized the response of irradiated chickens to the levels of untreated control.

3.3. Body weight

The body weight declined in all irradiated groups at a week after irradiation and normalized at the second week. SCE group showed a significant increase at first and second weeks after SCE administration, when compared to control group (Fig. 2).

3.4. Relative lymphoid organ weight

No significant differences in the relative lymphoid weight of the thymus, spleen and bursa between different groups were shown (Fig. 3).

4. Discussion

The results of the present study showed that chickens exposed to 600 rad whole-body X-radiation had highly impaired primary responses to a T-independent antigen such as BA but considerable

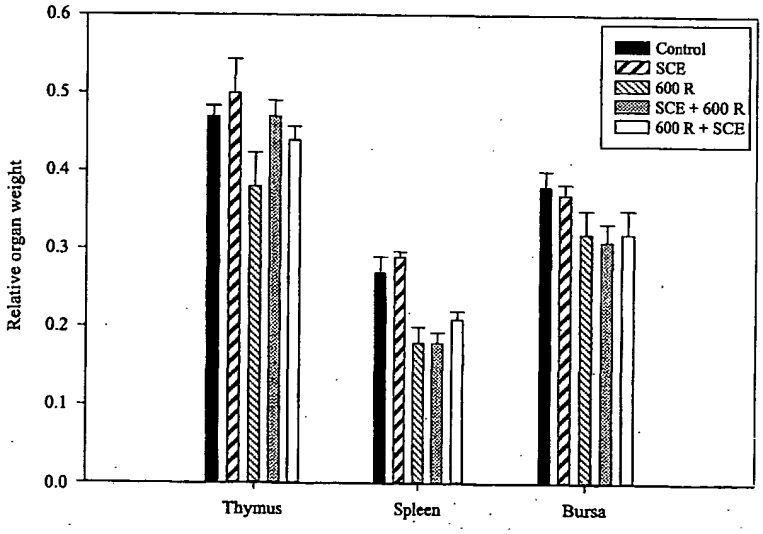

Fig. 3. Effects of SCE on the relative lymphoid organ weight of chickens. Three-week-old chickens were administered SCE at a dose of 500 mg/kg/day for three consecutive days. The thymus, spleen and bursa were excised free from adhering tissues and weighted individually. Values represent mean ± S.E.

responses to a T-cell dependent antigen such as SRBC. These results may be attributed to the difference in radiosensitivity among B-cell subpopulations, at least from the functional point of view. Although there is little information concerning the effects of sublethal irradiation on the immune responses of chickens to different antigens, selective immunosuppression in mice to various antigens after radiation has been reported [17–18]. Noteworthy, chickens in 600 R group showed almost normalized second immune responses, suggesting their recovery. On the other hand, they showed significant inhibition of DTH responses to HγG. A possible explanation of these results may be as follows. DTH is a T-cell-dependent response [19,20], which requires priming of a specific type of T cell (T_{DH}) upon sensitization (afferent limb) and efficient recruitment with release of mediators upon elicitation (efferent limb) [21]. On the other hand, DTH response is regulated by specific suppressor type of T cells (T_S) [21,22]. The hyporesponsiveness of the DTH response in irradiated chickens may suggest a defect at any of these phases and/or over down regulation by suppressor cells [21]. In some circumstances, DTH responses can be inhibited with enhancement of antibody response [23].

Oral administration of SCE significantly enhanced the first and second immune responses to SRBC and BA, in addition to sustaining DTH responses to HγG. Furthermore, those chickens exhibited significantly increased body weight at 4 and 5 weeks of age, when compared with untreated controls. These results are in agreement with our previous results [11,12], which showed enhancing effects of SCE on humoral antibody and DTH responses and body weight in chickens. Augmentation of humoral antibody responses by SCE may indicate to enhance the maturation of specific B cells, possibly as a consequence of stimulated proliferation and/or differentiation [1]. In addition, chickens administered SCE showed enhanced and prolonged antibody responses to commercially available *Salmonella* Enteritidis vaccine, showing adjuvant activity [11]. The adjuvant effect of SCE may be involved in the elevated synthesis of specific antibodies to SRBC and BA. Furthermore, the demonstrated effect of SCE on the promoting phagocytic activity of mononuclear and polymorphonuclear cells of chickens [12] may enhance the innate immunity and subsequently the host defense against opportunistic pathogens. Increased activities of phagocytes may enhance antigen processing and presentation and in turn, at least in part, augment the antibody responses [3,23]. Significantly increased body weight in SCE-administered chickens compared to untreated control may reveal growth-promoting activities of the extract [12].

Oral administration of SCE before or after irradiation restored the immune competence of the chickens as shown in both for humoral antibody production and DTH responses. El-Abasy [13] indicated the effects of SCE on histological and functional reconstitution of the lymphoid system of cyclophosphamide-treated chickens. In the same theme, many natural extracts showed immunostimulating activities in immunocompromised animals. Sharma and Ray [24] elucidated that septilin, a herbal preparation, enhanced primary and secondary responses in mice immunized with SRBC and inhibit the suppressive effect of prednisolone when orally administered with the drug. Pule bark extract restored phagocytosis and the immune response to SRBC in mice suppressed by prednisolone [25]. Immunosuppression due to old age or hydrocortisone treatment in mice overcame after administration of herbal preparation consisting from aqueous-ethanolic extract of mixed herbal preparation [26].

Although the exact mechanism(s) of the counteracting effect of SCE on the radiation-induced suppression in chickens remains open, antioxidants, free radical-scavenging activities and cytokines involved in cell proliferation and differentiation and haematopoiesis might be involved in the possible mechanisms. Noteworthy, Harris and Sljivic [27] concluded that the effects of whole body X-ray-irradiation on the plaque forming cell response of spleen of mice were not directly on the process of antibody synthesis by cells but on some more radio-sensitive process, namely, DNA metabolism. The mechanism of an enhancing effect of SCE in irradiated chickens may involve either direct or indirect effects to protect and/or repair the radiation-induced injury. Taken together, the results of the present study suggest that SCE can augment the outcome of acquired immune responses such as antibody production and DTH both in normal and X-irradiated chickens. Further studies are required to elucidate the mechanism(s) by which SCE exerts such activities.

In summary, the present study showed the efficacy of SCE as one of immunostimulants which can protect and/or recover from X-ray radiation induced immunosuppression in chickens.

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Article 4

PS-6108 鶏インターフェロンγのサルモネラ抗原に対するアジュバント活性

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【目的】インターフェロンγ（IFN-γ）は、Th1サイトカインとして知られ、サルモネラを始めとする細胞内寄生菌に対して効果的な防御の成立に関わることがマウスモデルで明らかにされている。我々は、鶏IFN-γ（ChIFN-γ）の大量培養・精製系を確立した。そこで、本サイトカインの*in vivo*での免疫アジュバントとしての効果を明らかにするため、本実験を行った。【方法】7週齢の鶏を10羽ずつ3群にわけ、1群には不活化*Salmonella* Enteritidis（1X10⁸CFU）を精製ChIFN-γと共に筋肉内に注射した。2群には不活化SEのみを、3群にはPBSを同様に注射した。免疫2週後にSE（1x10⁸CFU/ml）を経口投与で攻撃した。攻撃後から盲腸便を経時的に採取し、サルモネラの排菌を調べ、攻撃13日後には解剖して、臓器内及び盲腸内の菌数を調べた。なお、免疫時から毎週採血し、血清中の抗サルモネラ抗体価を凝集試験により測定した。【結果と考察】1群と2群には、免疫1週後から凝集抗体の出現が認められ、抗体価は両群で大きな違いはなかった。3群には免疫期間中、抗体の誘導は認められなかった。攻撃後、SE排菌数は3群で最も高く、1群で最も低かった。特に、攻撃1、4、10日後は、統計的に有意であった。また、臓器内菌数は、肝臓ではいずれの群にも菌が検出されなかったが、脾臓では1群と2・3群との間で有意差が認められた。以上をまとめると、ChIFN-γの抗原との同時投与は、凝集試験で見る限り、液性免疫には影響を与えなかったが、サルモネラの排菌・定着に対して抗原単独よりも効果的に防御に働き、細胞性免疫を誘導していることが示唆された。

PS-6109 さとうきび抽出物給与雛におけるサルモネラ・エンテリティディス排菌抑制について

○中村修，山本敏昭，田上雅之，水谷武雄[1]（日本配合飼料（株），[1]新三井製糖（株））

【目的】鶏卵を介したサルモネラ・エンテリティディス（SE）による食中毒は、世界的に公衆衛生上問題となっている。我々は「さとうきび抽出物」の免疫能向上効果に着目し、「さとうきび抽出物」10%製剤（本剤）を添加した飼料を雛に給与し、SE人工感染した場合のSE排菌抑制効果について検討した。【材料及び方法】試験1：1日令採卵鶏雄雛を1試験区当たり20羽（5羽ずつ4群）使用し、試験区は本剤を0.05%及び0.1%添加した飼料を、対照区は本剤無添加の飼料を47日令まで給与した。SEは5日令に1羽につき10⁸個を経口感染させた。また、44日令から3日間絶食，絶水処理によるストレスを負荷した。SEの測定には、盲腸便（13，27及び34日令）あるいは盲腸内容物（40及び47日令）を試料として用いた。試料は緩衝ペプトン水で前培養後、HTT培地で選択増菌培養し、DHL寒天に塗抹して黒色コロニーの性状よりSEであることを確認した(1回で検出されない試料については引き続き遅延二次増菌培養を実施した)。試験2：本剤の有効添加濃度を検討するため、0.1%，0.2%及び0.5%の添加濃度を設定して試験1と同様な方法で試験を実施した。【結果及び考察】試験1：40及び47日令に測定した0.1%添加区のSE陽性羽数は、対照区に比べ約半数に減少した。試験2：47日令に測定した0.1%区のSE陽性羽数は、他の試験区及び対照区に比べ、半数に減少した。試験結果から、本剤を飼料に0.1%添加することによりSEの排菌が抑制されることが明らかになった。

PS-6110 採卵育成鶏における生薬の*Salmonella* Enteritidis排菌抑制効果

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【背景】生薬が抗菌作用を有することは知られており、最近、食中毒細菌に対する抗菌作用も報告されている。今回、我々は数種類の生薬を鶏用飼料へ添加し、*Salmonella* Enteritidis（SE）に対する排菌抑制効果を調べた。【材料と方法】供試飼料として、ガジュツ、キキョウ、チョウジ、ショウキョウ、ケイヒ、ナンカシをそれぞれ0.1%添加した中雛用育成飼料、ガジュツとウコンを等量混合（二混）あるいはガジュツ、ウコン、キキョウを等量混合（三混）して0.1%添加した飼料を用いた。供試鶏として7週齢採卵鶏を用い、1群10羽とした。まず、予備飼育としてそれぞれの生薬添加飼料を1週間給与後、SE HY-1 10⁷CFU/mlを経口接種し生薬添加飼料の給与を継続した。接種1,4,7,10,14日後に盲腸便排菌数を計測し、14日後に剖検し肝臓、脾臓、盲腸内生菌数を調べた。【結果】ガジュツ、キキョウ、チョウジ添加区では著効を示し、無添加対照区に比べて有意な排菌抑制効果（3時点で$p<0.01$-0.05）が認められ、ショウキョウでは1時点のみ、また、二混では4時点、三混では二時点で有意（$p<0.05$）であった。一方、ケイヒ、ナンカシでは排菌抑制効果は認められなかった。また、盲腸内容、肝臓、脾臓の生菌数ではガジュツ添加区の盲腸内容以外では有意な差は認められなかった。【まとめ】生薬を単独であるいは混合して飼料に添加することによって、SE排菌数を有意に抑制出来た。このメカニズムについては今後の課題であるが、直接的な抗SE作用、あるいは間接的に正常細菌叢のバランスを変化させた結果としての抗SE作用等が考えられる。今後は実用化に向けてさらに検討して行きたい。

PS-6111 *Campylobacter jejuni* 排菌抑制効果の評価系について

○鈴木麻奈三，西村肇，西島宏紀，森腰俊亨[1]，竹原一明，中村政幸（北里大・家禽疾病，[1]伊藤忠飼料）

【背景】*Campylobacter jejuni* による食中毒は主としてブロイラー腸管内に生息する同菌の交差汚染によって鶏肉が汚染され、これが原因となっている場合が多い。このような交差汚染を防ぐためには、生産段階においてブロイラーの*C. jejuni* 汚染を減少させることも有効な手段と考えている。今回、本菌の排菌抑制効果評価法について検討するとともに数種の製品等で排菌抑制効果を調べた。【材料と方法】*C. jejuni* をナリジクス酸（NA）耐性にした。親株およびNA耐性株(NA株)を用いて以下の実験を行った。すなわち1群20羽の初生ブロイラーひなを搬入し、その日の夕方に親株とNA株をそれぞれ10^8 CFU/0.2ml経口接種し、1,2,3,4週後に1群5羽ずつ解剖し、盲腸内容、肝臓、脾臓の生菌数を求めた。増菌培地はCEM培地、分離培地はCCDA培地を用いた。実験1：親株とNA株の比較、実験2：NA株を用いたインテクリーン処理の効果（菌接種は2日後）、実験3：NA株を用いた0.015%マンノース飼料添加の効果、実験4：NA株を用いた抗*C. jejuni* 羊血清の効果(凝集反応抗体価1,280倍血清をPBSで100倍希釈し1日当り500ml/群を飲水投与)。【結果とまとめ】実験1において両菌株の盲腸内容生菌数は10^8 CFU/g前後で推移し有意差は認められなかった。したがって、本NA株は排菌抑制効果確認試験の指標菌として使用できるものと考えられた。実験2,3,4では排菌抑制効果は認められなかった。今後はこの系を用いて野外で使用できる有効な飼料添加物等について検討するつもりである。なお、いずれの試験においても、肝臓、脾臓ではほとんど増菌レベルの分離（3.6-20%）であり、供試菌株の侵襲性はサルモネラに比べて極めて低いものと考えられた。

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Translation of Article 4

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Suppression of Salmonella Enteritidis Excretion in Chicks Fed on a Sugar Cane Extract

Osamu Nakamura, Toshiaki Yamamoto, Masayuki Tagami, Takeo Mizutani

Nippon Formula Feed Mfg Co., Ltd and Shin Mitsui Sugar Co., Ltd

[Purpose]

Food poisoning caused by Salmonella Enteritidis, abbreviated as SE, through chicken eggs is a globally major problem to the public health. We have focused on an immunity enhancing effect of a "sugar cane extract" and fed chicks on feed supplemented with a formulation containing 10 % of the "sugar cane extract", present formulation, and studied on an effect of suppressing SE excretion in chicks artificially challenged with SE.

[Materials and Methods]

Test 1: Twenty male chicks of one-day age from egg-laying chickens, four groups of each five chicks, were used as one section. The chicks in a test section were fed on feed supplemented with 0.05 %

or 0.1 % of the present formulation, while the chicks in a control section were fed on feed without the present formulation until they became 47 days old. The chicks were challenged orally at the age of 5 days with 10 5 of SE per chick. Then they were subjected to abstinence from food and water for three days from the age of 44 days to put them in a stressed state. Cecum feces, at the age of 13, 27, and 34 days or contents of cecum at the age of 40 and 47 days were used as a sample for detecting SE. The samples were precultured in buffered peptone water, subjected to selective enrichment in an HTT medium, and then spread on a DHL agar plate to confirm, based on the properties of the resulting black colonies, that they were colonies of SE. (When no SE was detected in a first selection culture, the sample was subsequently subjected to delayed secondary culture.) Test 2: In order to determine an effective concentration of the present formulation to be supplemented, the same test was performed as in the test 1 now with the 0.1 %, 0.2 %, and 0.5 % of concentrations.

[Results and Discussions]

Test 1: The number of SE-positive chicks in the 0.1 % supplement section was about a half at the age of 40 and 47 days, compared to that of the control section. Test 2: The number of SE-positive chicks in the 0.1 % supplement section was about a half at the age of 47 days, compared to those of the other test sections

and the control sections. From the test results, it is seen that excretion of SE is suppressed by adding 0.1 % of the present formulation to feed.

(Published September 7, 2001)

Reference 5

Spencer-Meade

Cane Sugar Handbook

a manual for cane sugar manufacturers and their chemists

by GEORGE P. MEADE

Formerly, Manager Colonial Sugars Company, Gramercy, Louisiana;
former Member Board of Directors Cuban-American Sugar Company
(Parent Company of Colonial)

JOHN WILEY & SONS, INC., NEW YORK · LONDON

Photograph—Clewiston Factory, Florida
The Largest Cane Sugar Factory in Continental United States
(United States Sugar Corporation)

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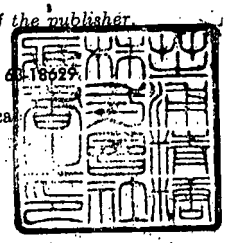
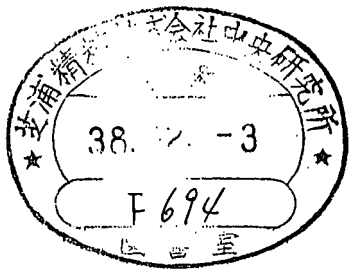
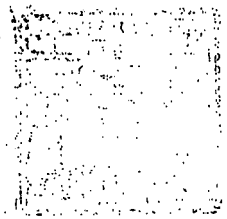




to Eleanore, for fifty years the ideal "Sugar Wife"



Reference 5

dling of imbibition juices (Fig. 5.13). Various methods for distribution of the juices to the blanket attempt to spread the flow of juice across the mill width: serrated-edged troughs, shooting the juice stream against a spray plate, overflow troughs, etc., none of which are very successful. Usual practice applies the water or juice to the bagasse as it issues from between the rolls with the idea that it will absorb the liquid like a sponge in expanding. The liquids do not penetrate to the lower layers of the blanket, no matter where or how applied.

The percentage of imbibition water varies with the country, the capacity of the mills, the character of the cane (especially its fiber content), and the relative costs of fuel and sugar. Modern mills and fuel economies have increased the amount of water permissible so that 25 to 30 percent is customary in larger factories where 10 to 15 percent was the rule 30 years ago. In Hawaii and Australia the range is from 25 to 40 percent.

5.20 Bath Maceration. Australia uses "true maceration" or "bath maceration." A description of the process by Harmon [25] follows:

> The system of hot maceration fluids and of diffusion baths or boots is one of the most distinctive features of Australian practice. The mills are spaced up to 60 feet apart and the bagasse from any one mill falls down a chute into a circular boot, kept overflowing with the macerating fluid. A large drum in this boot takes the chain of the intermediate carrier which is of the rake type with fingers above as well as below the chain attachment. A compound maceration system is employed but the maceration fluids are heated almost to boiling point before being sprayed on at the rollers. Varying quantities of maceration water are added, but in an efficient quadruple mill about 40 gallons water are added per ton cane (i.e., 18 percent on cane). Steam is also added direct to the diffusing boots and the temperatures of the mill-expressed juices other than the first are about 170° F. The saturated bagasse takes in most cases up to 10 minutes to travel from mill to mill and, although the crushing pressure is relatively low, as compared with Hawaii, this combination of diffusion (really lixiviation) and crushing results in an extraction of about 95 percent on the average, but ranging up to 98 percent in the best cases. The "lost juice" per 100 fiber is about 25.

The process is critically reviewed by Clayton,[26] and Hugot [27] concludes, "The gain in extraction is scarcely worth the complications involved." This conclusion is significant, as Jenkins (Hugot's translator-reviser) is from Australia.

5.21 Hot or Cold Imbibition Water. Whether to use hot or cold imbibition water is a matter of some controversy, but hot water (180° F.) is generally preferred throughout the world. The arguments for hot water are as follows: some slight fuel economies; rupture of some cells by heat (above 160° F.); some slight evaporation from the bagasse in transit; the use of return condensate from evaporator bodies; a slight gain in extraction, not always detectable.

[25] ISSCT. 1935, p. 45.

[27] *Hugot-Jenkins*, 1960, p. 243.

Honig says, "Hot imbibition has definite advantages in the efficiency of sugar extraction in the mills; by the use of hot imbibition water no unfavorable influence has been observed in the clarification." [28] Even under the best conditions the imbibition process is not entirely effective in that it does not dilute all the juice in the bagasse. The juice remaining is always higher in purity than that pressed out, although each successive pressure following the addition of imbibition water gives juice of lower purity.

5.22 Composition of Mill Juices. The juices from the crusher and each of the succeeding mills differ according to the pressure and the extent of saturation. With continued crushing a reduction occurs in the Brix, in the polarization, and in the purity, witn a consequent increase in the non-sugars both organic and inorganic. The following figures are from actual milling with compound saturation on a fifteen-roller mill and double crusher:

Source of Samples	Brix	Polarization	Purity
Double crusher	17.16	14.50	84.50
First mill			
Front roll	17.08	14.12	82.67
Back roll	16.13	13.06	80.97
Second mill			
Front roll	7.63	5.83	76.41
Back roll	9.37	7.31	78.01
Third mill			
Front roll	5.04	3.73	74.01
Back roll	6.14	4.54	73.94
Fourth mill			
Front roll	3.00	2.18	70.60
Back roll	4.52	3.26	72.12
Fifth mill			
Front roll	1.31	0.88	67.18
Back roll	2.55	1.78	69.80

In general, the juice from the back roll is of higher Brix and greater purity than that from the front or feed roll because the feed roll extracts the superficial imbibition water on the exterior of the bagasse particles, whereas the back roll extracts part of the juice in the inner cells. Hugot [29] adds that the feed roller of the later mills with wet crushing should furnish about three-fourths of the juice and the back roller only one-fourth.

In summary of the subject of the composition of the extracted juice, the more sucrose that is extracted (by ordinary means) the greater the proportion of undesirable material that will accompany the sucrose. It is a question not only of lower purities but also of the character of the impurities which reduce the purity.

[28] ISSCT, 1953, p. 716.

[29] *Hugot-Jenkins*, 1960, p. 256.

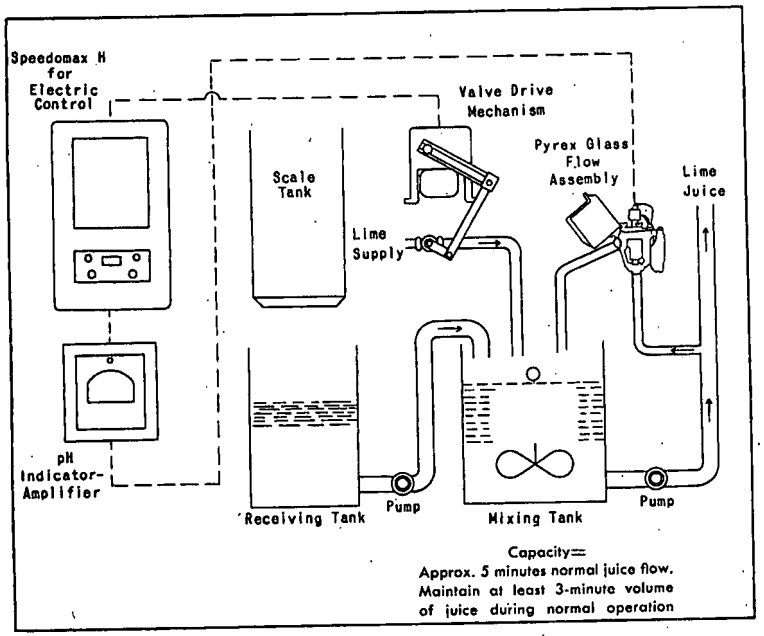


FIGURE 7.3. Automatic pH. (Leeds and Northrup)

automatic control of the addition of the lime will be described first. Payne [18] discusses the theory and practice of electrometric pH measurement, and later [19] the automatic control of pH for cane juice clarification. Davies [20] gives the practical considerations for electrometric pH control.

7.14 Automatic pH Control of Liming. Many instrument manufacturers both in the United States and abroad have systems incorporating pH-indicating and recording devices with actuating mechanisms that control the addition of lime to the mixed juice. Figure 7.3 shows one such assembly described by Leeds & Northrup, the manufacturers, as follows:

A sample of the limed juice is taken from the discharge side of the pump from the mixing tank. This sample passes through a flow chamber enclosing a glass measuring and a reference electrode which sense a voltage proportional to the pH of the limed juice. This millivoltage signal developed across the high resistance glass membrane is fed to a suitable indicating amplifier and thence to a servo-type potentiometric recorder which includes a control slidewire and immediately below a control relay which is further wired to the slidewire on the valve drive mechanism. As the control slidewire of the recorder changes position due

[18] *Honig I*, 1953, pp. 459–66.

[20] *Ibid.*, pp. 546–8.

to a change in the pH measurement, the follower slidewire in the valve drive mechanism is repositioned by a reversing motor actuated through the control relay unit. Thereby sufficient lime is added to maintain the final desired pH of the limed juice leaving the mixing tank.

The Bristol Company has another automatic pH regulator (Fig. 7.4) in which the electrometric method of measurement is described as follows:

The electrode assembly consists of a glass electrode, a reference electrode, and a thermo-compensator. The reference electrode maintains a constant potential independent of solution pH. The potential produced at the glass electrode is dependent upon the hydrogen-ion concentration in the electrolyte. The thermo-compensator adjusts the emf-pH relationship to compensate for temperature variations. The two electrodes constitute an electrolytic cell, the difference in potential between them producing an emf signal to the pH amplifier and then to the Dynamaster recorder or recorder-controller.

The electrode hood assembly in most of these systems is made of stainless steel and is of rugged construction. The method of proportioning the lime to the juice differs in the two diagrams shown in Figs. 7.3 and 7.4, as well as in other automatic control arrangements. In general, present-day electrometric pH controls for liming juice are reliable and troubleproof industrial units. All modern refineries control the addition of chemicals at the clarification station by automatic equipment.[21, 22] The automatic methods, particularly with continuous liming, have many advantages over manual controls.

7.15 Manual Control with pH Recorders. Electrometric pH recorders with manual control of the addition of the lime are less expensive than the fully automatic methods just described, but are not as satisfactory because of dependence on the skill and attention of the operator. The recorder, of a type similar to those illustrated in Figs. 7.3 and 7.4, should be located where the operator can see it at all times, preferably in the juice stream after thorough mixing of the lime. With batch liming methods this will give fairly satisfactory results, since the time element permits the operator to change the lime dosage in accordance with the pH readings. A further refinement would be a pH recorder on the clarified juice effluent, so that comparison of the pH before and after clarification would allow for the drop in pH described in Sec. 7.12. The Australian recommendation [23] says, "Since raw juices vary so widely in their buffer capacity, systematic tests on the clarified juice at frequent intervals provide the only means of manually controlling the liming satisfactorily."

The *Laboratory Manual for Queensland Sugar Mills* [24] provides an extensive discussion of electrometric methods and the construction details of various electrodes.

7.16 Temperature Range. The final temperature to which juices are heated in clarification varies from extremes of 194° F. (90° C.) to 238° F.

[21] See Alberino, SIT, 1961, p. 57.

[22] Gillette, BCRP, 1959, p. 225.

[23] *Queensland*, 1961, p. 140.

[24] *Ibid.*, pp. 133–40.

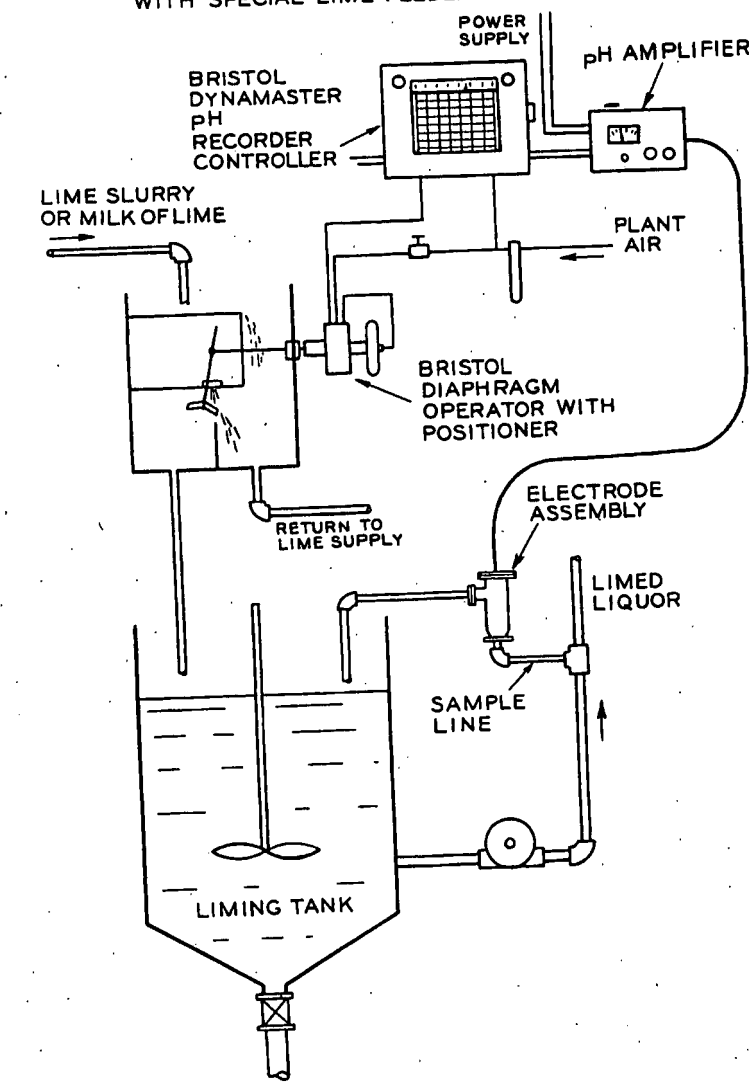


FIGURE 7.4. Bristol pH Control System.

(115° C.), although by far the commonest practice is to heat to slightly above the boiling point. Superheating (i.e., heating to well above the boiling point) was advocated by some in earlier studies, but the general opinion today is that superheating is not advantageous and that temperatures just above the boiling point—say 218° F. (103° C.)—are the maximum for good practice. For this reason the literature for and against superheating does not warrant review here.

7.17 Sequence of Liming and Heating. The classical simple defecation procedure is to add all the lime (by whatever method of control) to the cold juice, rapidly heat the limed juice to the final temperature (almost always to the boiling point or just above), and then settle by any of the means described in Chapter 8. This process, termed *cold liming*, is still used in many cane-growing areas, especially with added phosphate or added electrolytes as described later. As will be seen from succeeding paragraphs, many modifications of simple defecation have been successfully introduced.

One of the earlier modifications, developed about 40 years ago, is *hot liming*, still practiced quite generally, especially in Java, in which the raw juice is heated and the lime added after the juice has reached the final temperature. Certain colloids (albumin and hydrous silica) are precipitated with heat at the pH of raw juice. Less lime is needed (15 to 20 percent, according to Jenkins),[25] and more rapid settling is reported. Marches[26] describes hot-liming practice in Java and says that it has resulted in "tremendous progress" in establishing a constant pH difference (see Sec. 7.12) between the hot limed mixed juice and the clarified juice, whereas it is "practically impossible to obtain a constant pH of clarified juice" with cold liming. He recommends recording thermometers to control the juice at temperatures at which liming is done and a regular flow of juice to the liming tanks as essentials for maximum results in clarification, as well as an indicating pH meter as previously described. The details in Marches's discussion will prove valuable to those interested in hot liming.

Superheating juice [say to 238° F. (115° C.)], already discussed in Sec. 7.16, has also been recommended for hot liming, but the evident possibilities of destruction of reducing sugars at alkalinities above 8.0 pH and of inversion at lower pH levels are recognized, making the practice highly questionable.

Intermittent liming, in which a part of the lime is added to the cold juice to pH 6.1 to 6.4 and then heated to boiling point or slightly above with further liming to pH 7.4 to 7.8, is exemplified by the Ventura system used in the Philippines, and in modifications elsewhere. The purpose of these modifications is to gain the advantages of heating acid juice (colloid precipitation) with avoidance of possible disadvantages of hot liming (inversion and destruction).

7.18 Fractional Liming and Double Heating (FL and DH). The most successful of all the intermittent liming practices is that of Davies and colleagues,

[25] *I.S.J.*, November 1933, p. 420.
[26] ISSCT, 1956, Vol. II, p. 567.

In the majority of cases, the juice enters the first effect at a temperature lower than its boiling point, and, therefore, the evaporation will be less than 1 lb. per pound of steam used. However, as the partially evaporated juice is transferred to succeeding cells, which operate at progressively lower temperatures because of the flash, the evaporation will be more than 1 lb. per pound of vapor. The extent of these positive and negative variations depends on the temperature of the incoming solution, as compared with those in the evaporator, and the proportion of the solution which has to be evaporated. It is very seldom that there is a balance, and it has become customary to expect considerably less than the performance promised by Rillieux.

If each body of the multiple effect were supplied with a solution at its boiling temperature, then Rillieux's first principle would hold.

The second principle has been checked against heat balances and is substantially correct. By its use, great steam economies have been made possible. It is strange that it was not generally recognized until the beginning of the present century, and there are many sugar factories in operation, even today, where its use has been completely overlooked. Vapor pans and vapor heaters are based on Rillieux's second principle.

With the third principle, no engineer can have any quarrel, nor would he think of installing any steam-using apparatus without providing adequate venting.

11.25 The Heat Balance. Since Rillieux's first principle is too inaccurate for the requirements of today, other methods have been devised for arriving at the desired results. These involve a complicated series of fractions and to avoid this the writer (A. L. W.) devised a much simpler and more practical method involving the use of trial and error. The result is the so-called heat balance, which has profitably been employed during the past 50 years.

By this method the evaporator calculations follow an accounting procedure, in which everything is tabulated in columns to show the flow of heat and liquid through the succeeding bodies of a multiple effect. Each reaction is described briefly, so that it is possible to write down the entire flow for an evaporator on one sheet of paper. After this has been done, all the results are available in convenient form for use in design or analysis.

The trial and error comes in guessing at the amount of steam required for a given set of conditions and in following through with the calculations for the entire multiple effect. With this result in hand, another set of figures is made by correcting the weight of steam assumed in the first guess to compensate for the excess or deficit in the amount of evaporation required. Usually three trials are sufficient.

11.26 Boiling Point Rise. The boiling temperature of sugar solutions is greater than that of water at the same pressure or vacuum, and the increase is called the boiling point rise, usually abbreviated b.p.r. (also called boiling point elevation or b.p.e.). Study of b.p.r. is complicated, since the function varies with the purity. (See Sec. 12.18.) For purposes of evaporator calculations, the table below is sufficiently accurate. The b.p.r. is exactly proportional to the weight of solids dissolved per 100 parts of water, which corresponds to Brix/(100 − Brix). If a recheck is desired, it is necessary to get only one point in the straight line curve, and from this, the b.p.r. for all other densities can be read (see Fig. 11.10).

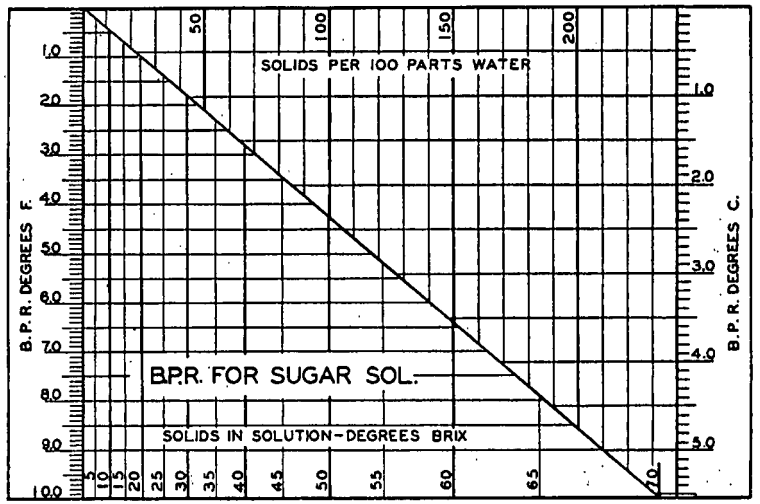


FIGURE 11.10. Boiling Point Rise for Sugar Solutions.

Degrees Brix	20.0	30.0	40.0	50.0	60.0	70.0
B.p.r., °F.	1.1	1.8	2.9	4.3	6.5	10.0
B.p.r., °C.	0.6	1.0	1.6	2.4	3.6	5.5

EXAMPLES OF SUGAR FACTORY CALCULATIONS

11.27 Practical Application. To make practical applications of the information outlined in the preceding pages an analysis will be made with the following sugar factory conditions assumed:

Cane ground per hour	50 tons
Weight of juice per hour	100,000 lb.
Temperature of raw juice	100° F.
Temperature of heated juice	218° F.
Temperature of defecated juice	205° F.
Brix of defecated juice	16.0
Brix of syrup	70.0
Purity of syrup	86.0
Exhaust steam available	9.0 lb. gage
Vacuum obtainable	26.0 (sea level)

form spontaneously. The liquid is concentrated to above saturation, after which a small quantity (a pound or so) of sugar dust is drawn into the pan. The dust does not serve as nuclei for grain but constitutes a shock to the supersaturated solution whereby the formation of new grain is induced sooner than in the older procedure. The shock should be applied as soon as saturation has been exceeded, which means in the metastable phase.

With hand control, the time to shock is determined by the string length of about 1 in. of a sample tested between thumb and forefinger. With instrument control the powder should be drawn in just beyond the saturation point, which can be taken at b.p.r. 16, or supersaturation 1.10 with 83 purity.

To shock too late involves the danger of the formation of conglomerates because of excessive concentration. The rate of sugar adsorption by any given crystal is proportional to its surface, which varies with the square of its linear dimension, whereas the volume or weight varies with the cube of its dimension. Hence the surface of a crystal as compared with its weight is inversely proportional to its size, so for a given weight of sugar the smaller the crystals, the greater the accreting surface and therefore the faster the growth. This corrects for initial grain irregularities, since the small ones grow faster than the large ones.

Irregularity of grain in the final strike may be caused by (1) a sudden increase in vacuum, (2) allowing evaporation to proceed too fast, (3) air leakage through the foot valve, and (4) the admission of cold feed into a hot pan while the strike is going up.

Grain will not appear immediately upon the introduction of powdered sugar. A minimum amount of air is admitted with the shock to avoid disturbing the temperature equilibrium. When grain begins to form, after a few minutes, it will be necessary to decide when to stop it by proof-stick examination as in the older practice.

Grain should be all in at b.p.r. about 19.0 (supersaturation around 1.40), so the change in concentration to revert to the metastable phase is not great. It seems better to effect this change by feeding syrup, and allowing the vacuum to remain undisturbed. Excellent results have been obtained in Hawaii by holding the vacuum constant during the entire boiling operation.

Until grain has been fully developed, the supersaturation should not go too high. Otherwise, even before false grain appears, conglomeration will take place, after which nothing can be done about it except to melt the sugar.

12.30 Pan Seeding. The best method of obtaining good grain establishment is by full "pan seeding" by adding at the proper moment the full amount of grain of predetermined size to equal the total number of grains in the finished strike. No grain is formed in the pan at any time and the concentration must be held in the crystal-growing, or metastable, phase. Seed is introduced as soon as the saturation point is reached, as indicated by instruments. To determine the proper amount of fines of a given grist to introduce into a pan to make a strike of sugar of a certain size, proceed as follows:

a. Find the weight of sugar expected from the strike.
b. Count and weigh about 500 crystals of this grade sugar.

c. Count and weigh 500 crystals of the seed powder to be used.
d. Dividing (*c*) by (*b*) and multiplying by (*a*) gives the weight of seed.

After this has been determined and tried, minor corrections can be made to take care of variations. Once the procedure has been established, standardization can eliminate the personal equation and the operation may be duplicated by anyone.[19]

This method of pan seeding is now used universally by refineries in the production of large-grain sugar such as sanding, manufacturer's standard, medium, and coarse. With the right amount and size of seed for the grain, these specialty sugars can be produced with a much more regular crystal, entirely free from conglomerates; this is practically impossible by the other methods.

It was mentioned above that the proper time to seed is when saturation is exceeded. With 83 purity and a strike temperature of 150° F., the b.p.r. at saturation is 14.5° F. Allowing a margin of 1.5, the proper seeding point would be at b.p.r. 16, at which the supersaturation is 1.10. To make this determination by hand, 1 gram of coarse sugar is placed in the notch of the proof stick, wetted with syrup in the pan, and withdrawn. If a magnifier shows the corners of the crystals rounded, saturation has not been reached. If the corners remain sharp and square, saturation has been exceeded and seeding or shocking may proceed. The saturoscope (Sec. 13.8) may also be used for this purpose. E. C. Gillett, in Chapter 13, considers factors affecting the rate of crystallization in massecuites. (See Secs. 13.17 *et seq.*)

12.31 False Grain and Conglomerates. Unless pan seeding is used, when the formation of grain is arrested by increasing the pan temperature, by dilution, or both, it is easy to overshoot the mark, dropping the mass below saturation and dissolving all the grain, which necessitates a fresh start. Even if the grain is not all destroyed, part of it may be, thus leaving an insufficient quantity and requiring additional nuclei to make up the loss. Fundamentally, after the grain has been obtained, the concentration must be brought back to the metastable or crystal-growing phase, where it must remain for the duration of the strike.

If the concentration is carried too high, false grain or smear will form and must be dissolved by dilution, preferably with water. Even before false grain appears, conglomeration may take place.

Conglomeration, mounted grain, married grain, rolled grain, all mean a grouping of a number of crystals which then grow together as one. Once formed, conglomerates will so remain to the end of the strike. These group crystals are objectionable because impurities and dirt lodge in the crevices, preventing proper washing and yielding a poor product of high color and low filtrability. In refined and direct-consumption sugars, the conglomerates lower the quality of the sugar as a whole and make thorough drying in the granulators more difficult.

[19] See also Webre, ISSCT, 1938, p. 945, for more detailed discussion of pan seeding.

CLINICAL VETERINARY MICROBIOLOGY

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Section 6: A Systems Approach to Infectious Diseases on a Species Basis

A microbiological approach to infectious diseases affecting domestic animals, including birds, is presented in Tables 168-174. These tables are organised on a species and systems basis. Species are dealt with in the following order: cattle (**Table 168**), sheep/goats (**Table 169**), pigs (**Table 170**), horses (**Table 171**), dogs (**Table 172**), cats (**Table 173**) and domestic birds (**Table 174**). The diseases listed under poultry may affect several avian species. Those conditions mentioned under turkeys, ducks, geese and pigeons are more common in, or specific to, these particular birds.

These tables are intended as a brief review of the major infectious diseases, including clinical aspects and diagnostic tests appropriate for each disease. Some of the diseases listed may occur infrequently in some geographical areas, whereas they may be endemic in other specific regions or countries.

Diseases where sudden death occurs do not lend themselves to description on a systems basis. Such diseases include anthrax and the clostridial enterotoxaemias. Lesions on the skin of the mammary glands and teats are covered in Chapter 36. Relevant information on chemotherapy relating to the bacterial and fungal diseases is presented in Chapter 7.

ABBREVIATIONS

+ve	positive
-ve	negative
Ab	antibody
Ag	antigen
AGID	agar gel immunodiffusion test
BA	blood agar
CAM	chorioallantoic membrane
CFT	complement fixation test
CNS	central nervous system
CO_2	carbon dioxide
CPE	cytopathic effect
CSF	cerebrospinal fluid
DCF	dilute carbol fuchsin stain
EM	electron microscopy
ELISA	enzyme-linked immunosorbent assay
FA	fluorescent antibody technique
HA	haemagglutination
HAI	haemagglutination inhibition test
IB	inclusion body
i/c	intracerebral
IFA	indirect fluorescent antibody test
IHA	indirect haemagglutination test (passive)
IN	intranuclear
i/p	intraperitoneal
i/v	intravenous
KOH	potassium hydroxide
Lab	laboratory
MAT	microscopic agglutination test
MZN	modified Ziehl-Neelsen stain
PM	postmortem
RIA	radioimmunoassay
SMEDI	stillbirth, mummification, embryonic death, infertility
TC	tissue culture
URT	upper respiratory tract
VN	virus neutralization test
ZN	Ziehl-Neelsen stain

Table 168. Infectious diseases of cattle.

BUCCAL CAVITY: cattle

Disease	Agent(s)	Comments	Diagnosis
ACTINOBACILLOSIS (Wooden or timber tongue)	*Actinobacillus lignieresii*	Wooden tongue is a characteristic form of the disease. A hard, tumorous mass develops in the substance of the tongue. There is anorexia, excess salivation and the tongue is hard and 'wooden'	• Clinical signs • Microscopy and culture if exudate is present
ACTINOMYCOSIS	*Actinomyces bovis*	Actinomycosis of the jaw can predispose to suppurative alveolar periostitis, often involving the fourth or third molars. The condition should be considered when loose cheek teeth occur in cattle	• Microscopy on sulphur granules in pus or exudate • Culture, if necessary
BLUETONGUE (BT)	*Orbivirus* (*Reoviridae*)	Infections are often subclinical or mild in cattle. Occasionally well-developed lesions in mouth, encrusted muzzle ('burnt' appearance), nasal discharge, laminitis (severe) and a patchy dermatitis are present	• History of endemic area • Virus isolation: i/v inoculation of 10-12 day old embryonated eggs • Serology: VN, AGID, ELISA, modified CFT
BOVINE PAPULAR STOMATITIS	*Parapoxvirus* (*Poxviridae*)	Papular and erosive lesions occur in buccal mucosa and on muzzle of animals under 6 months of age. No systemic involvement	• History of milkers' nodules in humans feeding calves • Lesions and no illness • EM on biopsy of lesions
BOVINE VIRAL DIARRHOEA (BVD) AND MUCOSAL DISEASE	*Pestivirus* (*Flaviviridae*)	BVD: Oral lesions are seen in about 75 per cent of animals with the diarrhoeal syndrome. There is diffuse reddening of mucosa, followed by focal lesions that develop into discrete, rounded, shallow erosions (1-2 cm) Mucosal disease: Erosive stomatitis is one characteristic sign. The oral lesions are discrete, rounded, sharply - defined depressions. Disease occurs in 12-36 month-old animals. There is also diarrhoea and often lameness. These animals are immunotolerant (Ab -ve) but have a persistent viraemia (Virus +ve)	• Clinical signs • FA on frozen sections • Virus isolation: buffy coat • Serology: ELISA or VN on paired sera
CALF DIPHTHERIA (Necrotic stomatitis)	*Fusobacterium necrophorum*	Predisposing cause may be rough foodstuffs. Usually seen in calves under 6 months-old. Necrotic lesions occur in buccal cavity or laryngeal region (dyspnoea)	• Clinical signs • Gram-stained smear on necrotic debris • Culture if necessary

Table 168. Infectious diseases of cattle. *(continued)*

BUCCAL CAVITY: cattle

Disease	Agent(s)	Comments	Diagnosis
DERMATOPHILOSIS (Streptothricosis)	*Dermatophilus congolensis*	Lesions can occasionally occur on the tongue as a result of the animal licking skin lesions	• Clinical • Giemsa-stained or Gram-stained smear of scabs • Culture, if necessary
EPHEMERAL FEVER (3-day-sickness)	*Rhabdoviridae*	Signs include apparent pain in the throat region, accompanied by dysphagia and hypersalivation. Some animals have impaired swallowing reflexes and if death occurs it is often attributable to inhalation pneumonia. Other signs include fever, sudden drop in milk production and lameness, either constant or shifting. Usual course is 1-3 days. Milk production may be depressed for that lactation	• Usually based on clinical signs • Haematology: neutrophilia is a constant finding • Serology: VN for rising Ab titre • Virus isolation: TC or mouse inoculation
GLOSSOPLEGIA **Botulism** **Listeriosis** **Actinobacillosis**	 *Clostridium botulinum* *Listeria monocytogenes* *Actinobacillus lignieresii*	Partial or complete loss of function of the tongue may be peripheral or central in origin. The aetiology can be traumatic or infectious. The unilaterally affected tongue is deviated towards the non-affected side. The bilaterally affected tongue is limp and protrudes from relaxed jaws	• Diagnosis of the specific condition and removal of any predisposing causes
IBARAKI DISEASE (Kaeshi disease)	*Orbivirus* (*Reoviridae*) Distinct from BT virus	Occurs in Far East and South East Asia. Acute arthropod-borne disease characterized by fever, ulcerative stomatitis and dysphagia that leads to dehydration and emaciation. Seasonal, in late summer and autumn	• Histopathology • Virus isolation in TC or fertile eggs and identification by VN
INFECTIOUS BOVINE RHINOTRACHEITIS	Bovine herpesvirus 1	Systemic disease in neonatal calves with rhinitis, conjunctivitis, erosions of soft palate, bronchopneumonia, often encephalitis and high mortality. In young adults the URT form is characterised by inflamed nares and ulcers in the nasal mucosa. In severe cases lesions occur in pharynx, larynx and trachea.	• Clinical signs • FA for Ag on frozen sections • Virus isolation
MALIGNANT CATARRHAL FEVER	Gammaherpesvirus	Hyperaemia and diffuse, superficial necrosis of oral and nasal mucosa are constant findings in this disease	• Clinical signs • Histopathology
RABIES (pharyngeal paralysis)	*Lyssavirus* (*Rhabdoviridae*)	Pharyngeal paralysis is usually a sign of encephalitis and occurs in cattle with rabies. The animal is unable to swallow, salivation is noticed with gurgling noises from the pharynx	• History of rabies being endemic • Clinical signs • Histopathology of brain: Negri bodies • FA (brain) : viral antigen

Table 168. Infectious diseases of cattle. *(continued)*

BUCCAL CAVITY: cattle

Disease	Agent(s)	Comments	Diagnosis
RINDERPEST	*Morbillivirus* (*Paramyxoviridae*)	Erosive stomatitis and gastroenteritis are characteristic for this disease. There are punched-out lesions on gums, lips, tongue and hard palate	• History of endemic area and clinical signs • AGID or CFT on lymph node biopsy for Ag • Histopathology • Virus isolation • Serology: CFT, ELISA, VN, HAI
VESICULAR DISEASES **Foot-and-mouth disease (FMD)** **Vesicular stomatitis (VS)**	*Aphthovirus* (*Picornaviridae*) *Vesiculovirus* (*Rhabdoviridae*)	Vesicular lesions on tongue, buccal mucosa, teats (milking cows) and interdigital cleft. Lesions on feet and teats less constant in VS than in FMD	• ELISA or CFT for Ag in vesicular fluid • Virus isolation • Serology: ELISA, CFT, VN

GASTROINTESTINAL TRACT: cattle

ANTIBIOTIC-INDUCED DIARRHOEA	*Pseudomonas* sp., *Proteus* sp., or *Candida albicans*	Calves treated for diarrhoea with a prolonged course of oral antibiotics. Normal flora destroyed predisposing to a chronic diarrhoea with poor response to treatment and progressive weight loss	• History • Isolation of secondary invaders from faeces in heavy growth
BOVINE VIRAL DIARRHOEA (BVD) AND MUCOSAL DISEASE	*Pestivirus* (*Flaviviridae*)	BVD: Young cattle 6-24 months show mild depression, oculonasal discharge and occasionally shallow ulcers in buccal cavity. Diarrhoea occurs in susceptible herds. High morbidity but zero mortality	• FA: frozen sections • Virus isolation: buffy coat • Serology: four-fold rise in Ab titre
		Mucosal disease: Low morbidity/100 per cent mortality. BVD-immunotolerant, 6-24 month-old animals at risk (virus +ve/Ab -ve). Severe lameness (laminitis), profuse diarrhoea and buccal cavity lesions extending to intestines occur	• Herd history and clinical signs • FA on buffy coat or lymphocyte smears for virus (virus +ve) • Serology: VN or ELISA for antibody (Ab -ve) • Virus isolation: buffy coat
CLOSTRIDIAL ENTEROTOXAEMIA	*Clostridium perfringens* types B and C	Occurs in young well-nourished calves up to 10 days of age. Severe haemorrhagic enterotoxaemia with rapid death. Uncommon	• Gross pathology and histopathology • Gram-stain on mucosa: large numbers of thick Gram +ve rods • Mouse tests for toxin in small intestine
COLIBACILLOSIS AND COLISEPTICAEMIA	*Escherichia coli*	Neonates under 7 days of age. Colostral immunity determines survival. Acute profuse diarrhoea, dehydration and acidosis	• Isolation of *E. coli* • Enteropathogenicity tests (*see* Chapter 18)

Table 168. Infectious diseases of cattle. *(continued)*

GASTROINTESTINAL TRACT: cattle

Disease	Agent(s)	Comments	Diagnosis
CRYPTOSPORIDIOSIS	*Cryptosporidium parvum*	Outbreaks of diarrhoea in 5-35 day-old calves. Both affected and clinically normal calves shed large numbers of oocysts in faeces. Villous atrophy and enlargement of crypts occurs	• Safranin-methylene blue stain on faecal smears • Auramine-O technique on faecal smear
JOHNE'S DISEASE	*Mycobacterium paratuberculosis*	Seen in cattle over 2 years of age, although infection is acquired soon after birth. Not all infected cattle become clinical. Chronic disease with emaciation, profuse diarrhoea and eventual death. Corrugated and reddened ileocaecal valve area is characteristic	• ZN smear of rectal scraping or mucosa of ileocaecal valve area • Culture: Herrold's egg-yolk medium : up to 16 weeks incubation • Histopathology
MALIGNANT CATARRHAL FEVER (MCF)	Ovine herpesvirus 2 (OHV 2) or Alcelaphine herpesvirus 1 (AHV 1, Africa).	Low morbidity/high mortality. Corneal opacity, enlarged lymph nodes of head and neck, ragged erosions in buccal cavity. Terminal diarrhoea and encephalitis. Reservoirs are sheep (OHV 2) and wildebeest (AHV 1)	• Histopathology • Virus isolation from buffy coat cells: calf thyroid cell line for wildebeest derived-MCF only
RINDERPEST	*Morbillivirus* (*Paramyxoviridae*)	High morbidity/high mortality. Very contagious. Profuse diarrhoea, dehydration, weakness, buccal erosions ('punched-out' ulcers) extending into the intestinal tract, 'zebra-striping' of terminal large intestine	• History and clinical signs • Detection of Ag in tissue: CFT, AGID • Histopathology • Virus isolation • Serology: VN, ELISA, CFT
ROTAVIRUS AND CORONAVIRUS INFECTIONS	*Rotavirus* (*Reoviridae*) *Coronavirus* (*Coronaviridae*)	Neonates 5-21 days old. Explosive outbreaks of profuse watery diarrhoea. Extensive villous atrophy (most severe with coronavirus). Calves that recover may be unthrifty until villi regenerate	• EM (faeces) • ELISA for Ag capture • Virus isolation and identification
SALMONELLOSIS	*Salmonella* spp.	Animals of all ages are susceptible. Young calves often develop the septicaemic form of disease. May be stress-induced. Acute diarrhoea / dysentery and fever are present. Deaths can occur in young animals	• Culture for salmonellae
WINTER DYSENTERY ('Black scours')	*Coronavirus*	Once thought to be due to *Campylobacter jejuni*. Explosive outbreaks of diarrhoea/dysentery in mature housed cattle. Outbreak lasts 24 hours, is usually non-febrile and there are dark watery faeces with a fetid odour. High morbidity / low mortality	• Virus isolation • Virus detection in faeces: EM, passive haemagglutination • Serology: VN, HAI

Table 168. Infectious diseases of cattle. *(continued)*

LIVER: cattle

Disease	Agent(s)	Comments	Diagnosis
BACILLARY HAEMOGLOBINURIA	*Clostridium haemolyticum*	Ingested spores lodge in the liver. Migrating liver fluke cause tissue damage and provide conditions suitable for germination of spores. Sudden death or fever, abdominal pain, 'port-wine' urine and infarcts in liver. The infarcts are pale, raised and surrounded by a bluish-red zone. Disease affects cattle and occasionally sheep	• History of a liver fluke area • Clinical or postmortem findings • FA on smears from liver lesion
BOVINE LIVER ABSCESSES	*Fusobacterium necrophorum* and *Actinomyces pyogenes*	Usually no clinical signs but the lesions are discovered at slaughter. Most common in feedlot cattle. Similar lesions have been reported in pigs	• Pathology • Direct microscopy: Gram-stained smear • Culture of pathogens
FACIAL ECZEMA (mycotoxicosis)	Sporidesmin in spores of *Pithomyces chartarum* (mycotoxin)	Bile duct obstruction, liver fibrosis, jaundice and failure to excrete phylloerythrin results in photosensitization seen on unpigmented or bare skin	• Clinical signs • Spore count on pastures • Gross and histopathology of livers
LISTERIOSIS (SEPTICAEMIC VISCERAL)	*Listeria monocytogenes*	Occurs in many species of young animals and in birds. There is fever, anorexia, depression and death in 1-3 days. Necrotic foci are seen throughout the liver and other body organs at postmortem examination.	• Direct Gram-stained smears from lesions • Culture of pathogen
RIFT VALLEY FEVER	*Phlebovirus* (*Bunyaviridae*)	Hepatitis and high mortality occurs in lambs, kids and calves; severe disease and abortions in adult sheep and goats, but only mild or subclinical infections in cattle with a high percentage of abortions. Vector: mosquitoes	• History of endemic area • Clinical signs • Gross and histopathology • Virus isolation: TC or i/p mouse inoculation • Serology: VN, CFT, ELISA, or HAI

Table 168. Infectious diseases of cattle. *(continued)*

GENITAL SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
AKABANE DISEASE	*Bunyavirus* (*Bunyaviridae*)	Japan, Australia, South Africa and Israel. Cattle, sheep and goats. • Severe damage to foetus with death and abortion • Congenital abnormalities: hydranencephaly and arthrogryposis Solid immunity after infection Vector: mosquitoes	• Virus isolation: suckling mice or TC • Serology: VN
ANAPLASMOSIS (Gall Sickness)	*Anaplasma marginale*	Arthropod-transmitted disease of ruminants in tropics and subtropics. Clinical disease seen in introduced, non-immune, adult cattle: fever, anaemia, icterus (but not haemoglobinuria), weakness and abortion in pregnant animals. Death, recovery or chronic disease with emaciation may ensue	• History of endemic area • Giemsa-stained blood smears for rickettsiae • Serological tests for carriers and chronic cases
BLUETONGUE	*Orbivirus* (*Reoviridae*)	Sheep, deer and cattle are affected but only about 5 per cent of infected cattle show clinical signs. If cattle are infected during gestation:- • Abortion • Congenital abnormalities: cerebellar hypoplasia, arthrogryposis or hydranencephaly Vector: *Culicoides* spp.	• Isolation: i/v inoculation of 10-12 day embryonated eggs • Serology: ELISA, VN, AGID
BOVINE GENITAL CAMPYLOBACTERIOSIS (Vibriosis)	*Campylobacter fetus* ss. *venerealis.* (*C. fetus* ss. *fetus*)	Venereal infection, bulls are carriers. Non-immune cows suffer mild metritis, salpingitis and embryonic death with irregular cycles at 28-35 days. Self-limiting disease and natural immunity in 3-5 months with destruction of *Campylobacter.* Occasional carrier cow (vagina). Sporadic abortions with *C. fetus* ss. *fetus*	• Isolation of pathogen from abomasal contents of foetus • Direct microscopy using DCF or FA

Table 168. Infectious diseases of cattle. *(continued)*

GENITAL SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
BOVINE VIRAL DIARRHOEA (BVD)	*Pestivirus* (*Flaviviridae*)	Syndromes include: • Neonatal calves: immunosuppression • Young cattle 6-24 months: diarrhoea and erosions of buccal mucosa • Adult pregnant cows, depending on stage of gestation when infected: a. 50-100 days: foetal death and abortion or mummification b. 100-150 days: congenital defects in foetus c. Before 120 days of pregnancy (with non-CPE strain): immunotolerance (virus +ve/Ab -ve) and calf is at risk from mucosal disease when 6-24 months old d. Virus via coitus: fertilization failure and a 'repeat breeder' problem	• Virus isolation • FA: frozen sections (Ag +ve) • Serology: VN, IFA, ELISA (Ab -ve)
BRUCELLOSIS	*Brucella abortus* (*B. melitensis* and *B. suis*)	Abortion storms occur in non-immune herds. A cow usually only aborts once but remains infected and excretes brucellae at subsequent parturitions. Organisms can be excreted in milk. Infection is usually by oral route. Notifiable disease in many countries	• Serology: many tests used on a herd basis as part of a national eradication scheme • Isolation of brucellae • Direct microscopy: MZN-stained smears
CHLAMYDIAL ABORTION	*Chlamydia psittaci*	Similar to enzootic abortion of ewes	• Isolation • Impression smears from cotyledons • Serology: CFT, IFA, ELISA
EPIZOOTIC BOVINE ABORTION (Foothill Abortion)	*Borrelia* sp.?	Abortion or full-term weak calves. Vector is *Ornithodoros* tick. No signs of illness in cows but abortions in 10-90 per cent of susceptible cattle in third trimester. Incubation period 90-150 days. Cows usually abort only once and are normal at next pregnancy. Occurs in Western USA	• Gross and histopathology. • Microscopy: darkfield (foetal blood)

Table 168. Infectious diseases of cattle. *(continued)*

GENITAL SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
INFECTIOUS BOVINE RHINOTRACHEITIS/ INFECTIOUS PUSTULAR VULVOVAGINITIS (IBR/ IPV)	Bovine herpesvirus 1.	IBR: Incubation period 2-6 days. Syndromes include: • Young adults: respiratory disease ('red nose') • Abortions up to 90 days after infection with or without previous respiratory signs. Abortion most common between 4-7 months of pregnancy. Infertility is not a sequel of IBR. No gross lesions in foetus but microscopic foci of necrosis in many organs with IN inclusions • Neonates: diarrhoea and/or encephalitis IPV: venereal infection, localized in genitalia of both sexes. No viraemia occurs so abortion is not seen. Self-limiting infection. Many subclinical cases occur	• Histopathology (foetal tissues) • Virus isolation: vaginal and preputial swabs • FA: foetal tissues • Serology: VN or ELISA
LEPTOSPIROSIS	*Leptospira interrogans* serovars	Abortion storms common with some serovars but are sporadic with others. Abortion occurs 6-12 weeks after infection and is most common in 7th month of pregnancy. Other signs include infertility, weak calves and an agalactia syndrome. Carrier state common with leptospires excreted in urine	• Darkfield or FA microscopy on urine • Culture from urine or kidneys • Serology (herd basis): MAT, CFT, ELISA
LISTERIOSIS	*Listeria monocytogenes* (*L. ivanovii* : abortion in cattle and sheep only)	Syndromes include: • Visceral or septicaemic listeriosis in many young animals and birds • Neural listeriosis (circling disease) in cattle, sheep and goats • Abortion in cattle, sheep and goats. Usually sporadic and in late gestation. No systemic illness in dam and no infertility. Listeriae are shed in milk and uterine discharges for some months after infection. Minor outbreaks occur with silage-feeding to pregnant animals • Ocular form: a self-limiting iritis often with corneal opacity. Associated with silage feeding Circling disease and abortions do not usually occur together on same property. Focal necrosis of foetal liver occurs but may be masked by autolysis	• Isolation and identification of pathogen • Histopathology

Table 168. Infectious diseases of cattle. *(continued)*

GENITAL SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
MYCOTIC ABORTION	*Aspergillus fumigatus,* or *Mortierella wolfii*	Abortions sporadic and usually between 6-9 months of pregnancy. Characteristic findings: • 'Wooden' appearance of placenta as some maternal caruncles detach and adhere to cotyledons • Ringworm-like lesions on foetus: pathognomonic when present • Placenta often retained *M. wolfii* abortions (in 5 per cent cases) can be followed, within 48 hours by peracute pneumonia and death of cow	• Histopathology on cotyledons or foetal lesions • Isolation of fungal pathogen
RIFT VALLEY FEVER	*Phlebovirus* (*Bunyaviridae*)	Epidemics in cattle, sheep and goats in South and East Africa. • Hepatitis and high mortality in young animals • Severe disease and 90-100 per cent abortions in sheep and goats • Mild disease in cattle but 100 per cent abortion rate • Influenza-like disease in man Vector: mosquitoes. Reservoir: wild ruminants	• Histopathology: liver necrosis. • Virus isolation: lab. animal inoculation or TC • Serology: VN, CFT, ELISA, HAI
SALMONELLOSIS	*Salmonella* species (especially *S.dublin*)	Sporadic abortions and the cow may or may not show systemic illness. Many abortions may be seen in a herd where an outbreak of enteric disease has occurred. Faecal-oral transmission and carrier state is common. Stress can convert a carrier state to a clinical case	• Isolation of salmonellae from placenta or foetus
TICK-BORNE FEVER	*Ehrlichia (Cytoecetes) phagocytophila*	Seen in Western Europe and Finland. Relatively mild enzootic disease with dullness, fever, and immunosuppression. Abortions and stillbirths in cattle and sheep. Rickettsial organism has predilection for neutrophils. Vector: *Ixodes ricinus*	• Direct microscopy. Giemsa-stained blood smear: purple bodies 0.3-0.7μm
TRICHOMONIASIS	*Trichomonas foetus*	Early embryonic death (infertility) and occasionally abortions or pyometra. Venereal infection: bull carrier in prepuce and can infect 90 per cent of cows served. Foetus dies 50-100 days after conception and irregular oestrous cycles follow. Uterine discharge often purulent and contains large numbers of protozoan parasites (maximum numbers 3-7 days before oestrus)	• Direct microscopic examination of uterine discharges. Specimens should be kept warm or protozoan parasite will become non-motile • Culture

Table 168. Infectious diseases of cattle. *(continued)*

URINARY SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
ABSCESSES IN KIDNEYS	Pyogenic bacteria such as: streptococci, *Staphylococcus aureus*, *Actinomyces pyogenes*	If a bacteraemia occurs, these pyogenic bacteria can lodge and multiply in the kidneys and in other body organs	• Isolation of the pathogenic bacteria • Histopathology
BACILLARY HAEMOGLOBINURIA	*Clostridium haemolyticum*	Associated with liver damage due to migrating liver fluke. The urine is 'port-wine' coloured and foams when voided. Sudden deaths are common. On postmortem, the liver infarct is pathognomonic: raised, light in colour and outlined by a bluish-red zone of congestion	• History: liver fluke area • Clinical signs • Pathology • FA on smears from liver lesions • Isolation of pathogen • Demonstrate toxin in peritoneal cavity fluid
BOVINE LEUKOSIS	*Retroviridae*	Tumours (B lymphocyte infiltration) can occur in the kidneys and in other body organs	• History of the disease in the herd • Clinical signs • Histopathology (dead animals or biopsy) • Serology: AGID or ELISA (herd test)
LEPTOSPIROSIS	*Leptospira interrogans* serovar *pomona* and other serovars	Some serovars such as *pomona*, produce a haemolysin resulting in haemoglobinuria in calves and occasionally older animals. There is usually accompanying fever, icterus and anorexia	• Clinical signs • Urine for dark-field microscopy • FA technique on urinary deposits • Isolation of leptospires
PYELONEPHRITIS (BOVINE)	*Corynebacterium renale* group (Streptococci and other bacteria often present)	Disease of mature cows. Often precipitated by pregnancy and dystocia. Urine cloudy with blood clots in advanced cases. There is frequent micturition, uneasiness, hunched back and enlarged kidney may be felt on palpation	• History and clinical signs • Isolation of a member of the *C. renale* group
'WHITE SPOTTED KIDNEY' IN CALVES	*Escherichia coli*, *Leptospira interrogans* serovars or other bacteria	Focal interstitial nephritis following a bacteraemia or septicaemia. Often only detected at slaughter	• Isolation of the pathogens may be difficult at this stage in the disease • Fixed tissue for histopathology may assist in the diagnosis

Table 168. Infectious diseases of cattle. *(continued)*

EYES AND EARS: cattle

Disease	Agent(s)	Comments	Diagnosis
INFECTIOUS BOVINE KERATOCONJUNCTIVITIS (IBK)	*Moraxella bovis*	Predisposing causes are irritants, flies and sunlight. Acute disease with highest incidence in animals under 2 years of age. Initial signs are photophobia, blepharospasm, lacrimation and conjunctivitis. Ulcers, corneal oedema and opacity occur with vascularisation in severe cases. Healing stage involves granulation tissue projecting from the ulcer as a characteristic 'red-cone'. Condition resolves completely or leaves a white corneal scar	• History of outbreak • Clinical signs • Isolation of *M. bovis* from lacrimal secretions (within 2 hours of collection)
INFECTIOUS BOVINE RHINOTRACHEITIS	Bovine herpesvirus 1 (BHV 1)	Conjunctivitis occurs as part of the acute syndrome and in mild cases may be the only sign present. In acute and severe disease, signs include fever, depression, nasal discharge and inflamed nares ('red nose'). Ulcers develop in the nasal mucosa. There is dyspnoea, mouth breathing and excessive salivation	• Clinical signs • FA: frozen sections • Virus isolation
LISTERIOSIS (corneal opacity)	*Listeria monocytogenes*	Corneal opacity is often unilateral when it occurs in neural listeriosis. Listerial iritis can be associated with feeding of big bale silage. The condition may progress to corneal opacity and blindness. Systemic signs are usually absent	• History of silage feeding • Clinical signs • Attempted isolation of listeriae from eyes in ocular form
MALIGNANT CATARRHAL FEVER (corneal opacity)	Ovine herpesvirus 2 or Alcelaphine herpesvirus 1	Bilateral corneal opacity is a constant finding in this sporadic but usually fatal disease	• Clinical course of disease • Histopathology
MYCOPLASMAL CONJUNCTIVITIS	*Mycoplasma bovoculi*	Causes conjunctivitis and transient corneal opacity. A concurrent infection with *Moraxella bovis* may increase the severity of IBK	• Isolation of *M. bovoculi*

Table 168. Infectious diseases of cattle. *(continued)*

NERVOUS SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
ABSCESSES (SPINAL OR BRAIN)	*Actinomyces pyogenes*, *Fusobacterium necrophorum*, *Actinomyces bovis*, or *Mycobacterium bovis*	Usually occurs in young animals. May arise from direct trauma such as dehorning or as an extension of otitis media, paranasal infections or lesions of the meninges. There is rotation or deviation of neck, ataxia, circling, blindness or nystagmus in one eye	• Gross pathology • Histopathology • CSF: high neutrophil count • Isolation of pathogen from CSF or lesion
BOTULISM	*Clostridium botulinum*	History of cattle eating toxin-containing foods (baled silage, processed poultry litter or carrion), but wound infections (toxicoinfections) can occur. Toxin causes progressive muscular paralysis with dysphagia, recumbency and respiratory failure. There is no fever. Mild cases may recover	• Type of foodstuffs • Clinical signs • Demonstration of toxin in serum: mouse inoculation or ELISA
BOVINE LEUKOSIS	*Retroviridae*	Tumours can occur anywhere in the body including the brain or spinal canal. Clinical signs will depend on the site of the tumour.	• Serology: AGID or ELISA on a herd basis • Histopathology
BOVINE SPONGIFORM ENCEPHALOPATHY (BSE)	A prion (probably the scrapie agent)	Highest incidence in adults 3-6 years old. Long incubation period 2-8 years. Onset is insidious. There is apprehension, low head carriage, irritability and excessive ear movement. Later ataxia, falling and recumbency occur. Occasionally there is aggression towards other animals. Pruritis is very rare. Course usually 1-3 months	• Clinical signs • Age • Histopathology: astrocytosis, vacuolation of neurons • EM: scrapie-associated fibrils in brain tissue
CONGENITAL CNS LESIONS	Akabane disease virus	Hydranencephaly and/or arthrogryposis	• Serology: test dam for high antibody titres to the appropriate virus • Virus isolation
	Bovine viral diarrhoea virus	Hydranencephaly, hydrocephalus, cerebellar hypoplasia, cataract or arthrogryposis	
HEARTWATER	*Cowdria ruminantium* Vector: *Amblyomma* ticks.	Endemic in southern Africa, Malagasy and some West Indian islands. Clinical signs occur in non-immune animals: lip-licking, high-stepping, recumbency and death during a galloping convulsion. Course of the disease is 3-6 days	• History of endemic area • Giemsa-stained smears of cerebral grey matter to visualise rickettsiae

Table 168. Infectious diseases of cattle. *(continued)*

NERVOUS SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
INFECTIOUS BOVINE RHINOTRACHEITIS (IBR)	Bovine herpesvirus 1	Infection in neonatal calves can cause conjuntivitis, pneumonia and nervous signs such as excitement, tremor, ataxaxia and recumbency. A severe non-suppurative meningoencephalitis/myelitis with marked vascular cuffing and gliosis is present. A strain of BHV-1 has been isolated from encephalitis in adults	• Clinical signs • Histopathology: tissue damage and IBs • Virus isolation • FA: frozen sections
LISTERIOSIS	*Listeria monocytogenes*	Neural form: occurs in all ages but most common in adults. Signs include drooling, facial hypalgesia, head tilt and unilateral drooping ear. Loss of blink reflex can lead to keratitis and corneal ulceration. Ataxia, circling and occasionally mania and bellowing occur. Course is less than 14 days. Other syndromes are abortion, septicaemic (visceral) form in young animals, and ocular form	• History and clinical signs • Histopathology: microabscesses and perivascular cuffing in brain • Isolation of listeriae from brain (cold enrichment)
LOUPING ILL	*Flavivirus* (*Flaviviridae*)	The disease in cattle is usually mild and seen mainly in calves, as adults acquire an immunity in endemic areas. Signs include excitement, tremors, incoordination and ataxia. A non-suppurative meningoencephalitis is present mainly affecting the lower brain stem and cerebellum. Vector: *Ixodes ricinus*	• History of endemic area with vector present • Clinical signs • Histopathology • Virus isolation: TC or i/c inoculation of mice • Serology: HAI, AGID, CFT, VN, IFA
MALIGNANT CATARRHAL FEVER	Ovine herpesvirus 2 or Alcelaphine herpesvirus 1 (Africa)	Sporadic disease, usually in adults. Reservoir is sheep (OHV 2) or wildebeests (AHV 1). Generalised disease with fever, encrusted muzzle, diffuse erosions in buccal cavity, corneal opacity, cervical lymphadenopathy, deep depression, incoordination, head-pressing and eventually paralysis and death. Mortality 100 per cent. A non-suppurative encephalomyelitis is present	• History of contact with sheep or wildebeests • Clinical signs: sporadic cases and usually fatal • Histopathology • Virus isolation: AHV 1 but not OHV 2 from buffy coat
MENINGITIS (BACTERIAL)	*Staphylococcus aureus*, *Haemophilus somnus*, *Escherichia coli*, Streptococci or others	Seen in young calves and condition usually results from a bacteraemia following infection of the umbilicus. There is fever, neck rigidity, opisthotonos, nystagmus, extensor spasms, clonus and coma. Glairy thickening of meninges and congestion of vessels occurs	• Histopathology • Isolation of pathogen from CSF or meninges

Table 168. Infectious diseases of cattle. *(continued)*

NERVOUS SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
POLIO-ENCEPHALOMALACIA (Cerebrocortical necrosis)	Attributed to thiaminases produced by bacteria in the rumen	Thiamine deficiency. Animals are afebrile, have decreased mobility, blindness with active pupillary and corneal reflexes, recumbency with extensor spasms and 'paddling' can occur. Rapid response to thiamine early in condition	• Clinical signs • Swift response to thiamine • Histopathology: focal necrosis of cortex
PSEUDORABIES	Porcine herpesvirus 1	History of close contact with pigs or rats. Signs include intense focal pruritis often in flank area, dog-sitting position, bellowing, teeth grinding, salivation, pharyngeal paralysis but no aggression is seen. Death from respiratory or cardiac failure	• Virus isolation • FA on cryostats of brain tissue • History: association with pigs
RABIES	*Lyssavirus* (*Rhabdoviridae*)	The dumb form is most common in cattle with salivation, tenesmus, constipation, ataxia and paralysis. Rarely the furious form is seen with bellowing, mania and aggression. A non-suppurative encephalitis is present with Negri bodies in neurons of cerebellum and hippocampus	• History of dog or fox bites (vampire bats in South America) • Clinical signs • FA on brain • Histopathology of brain for Negri bodies
SPORADIC BOVINE ENCEPHALOMYELITIS (Buss disease)	*Chlamydia psittaci*	Described in USA, Europe, Japan, Australia and South Africa. Incubation period 6-31 days. Chlamydiae excreted in faeces and urine. Depression, fever, salivation and dyspnoea. Recovery can occur at this stage but CNS signs usually develop: stiff, staggering gait, circling and falling over small obstacles. Limbs become weaker and general paralysis follows. Low morbidity but mortality >50 per cent. All ages affected but most common in young animals. In chronic cases, serofibrinous exudates occur in body cavities. Course of disease usually 10-14 days	• Pathology: peritonitis • Isolation: yolk sac or TC • Serology: group antigen. Rising titre, CFT or ELISA
TETANUS	*Clostridium tetani*	Spores enter via traumatised tissue following dystocia, wounds, injections and umbilicus. Signs include muscle stiffness ("saw horse" stance), tremors, trismus, hyperaesthesia, raised tail-head, bloat, tetanic convulsions, opisthotonos and death from respiratory paralysis	• History: predisposing cause • Clinical signs • Gram-stained smear from deep wound if present

Table 168. Infectious diseases of cattle. *(continued)*

NERVOUS SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
THROMBOEMBOLIC MENINGOENCEPHALITIS (TEME)	*Haemophilus somnus*	Septicaemic form of infection ('sleeper syndrome') is seen most often in young feedlot cattle in autumn and winter. There is fever, stiffness, extension of head, lingual paralysis, ataxia, stupor, opisthotonos, occasional circling and blindness. Haemorrhagic infarcts occur in brain and retina	• Clinical signs • Gross pathology of brain • Histopathology • Isolation of *H. somnus*
TREMORGEN STAGGERS	*Penicillium* or *Aspergillus* spp.	Often seen in calves. There is stiffness, ataxia, trembling in large muscle masses, falling and convulsions if hurried. Animals should be moved slowly and gently from suspect pasture	• Clinical signs • Recovery on removal from suspect pasture
VERTEBRAL OSTEOMYELITIS	*Salmonella* spp. *Actinomyces bovis*	Signs dependent on site of lesion. Ataxia, hemiplegia or paraplegia can occur due to either pressure on spinal cord or extension to and inflammation of the spinal meninges	• Gross pathology • Isolation of pathogen

MUSCULOSKELETAL SYSTEM: cattle

BLACKLEG	*Clostridium chauvoei*	Sudden death usually occurs, especially if heart muscle is involved. Muscle masses of hind quarters commonly affected and age range is 3-24 months. The muscles are dry, dark, spongy with small gas bubbles and have a sweet, rancid odour. Crepitation can be felt. Usually an endogenous infection in cattle	• History: endemic area • Clinical signs • FA on muscle or bone marrow from a rib • Isolation of pathogen, if necessary
BOTULISM	*Clostridium botulinum*	Usually an intoxication from toxin-containing baled silage, processed poultry litter or carrion-eating. Less commonly wounds are contaminated by spores and toxicoinfectious botulism occurs. Signs include progressive weakness, tongue paralysis, inability to swallow, flaccid paralysis and death from cardiac or respiratory paralysis	• History of foodstuffs • Clinical signs • Demonstration of toxin in serum by mouse inoculation
CHLAMYDIAL POLYARTHRITIS	*Chlamydia psittaci*	Can involve all ages but calves 4-30 days of age are most severely affected. Lameness is pronounced but calves remain alert and will suck if aided. Limb joints are swollen and painful. There is no navel involvement	• Clinical signs • Cytological examination of joint fluid for elementary bodies or inclusions • Isolation of pathogen

Table 168. Infectious diseases of cattle. *(continued)*

MUSCULOSKELETAL SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
CONGENITAL DISEASES **Bluetongue** **Akabane** **Bovine viral diarrhoea**	*Orbivirus*, *Bunyavirus*, *Pestivirus*	Congenital abnormalities can occur if each of these viruses infect cows at a critical point in gestation. In all three infections, common abnormalities in the calves include arthrogryposis and hydrocephaly	• Serology: detection of antibodies to each of these viruses in the dam • Virus isolation
EPHEMERAL FEVER	*Rhabdovirus*	Characterised by a sudden onset, biphasic fever, drop in milk production, depression, muscle stiffness and lameness. Usually recovery is dramatic and complete in 3 days ('three-day-sickness'). Vectors are mosquitoes and *Culicoides* spp.	• Clinical signs in an endemic region • Haematology: neutrophilia • Serology: VN (specific) or IFA • Virus isolation: TC or mouse inoculation
ERGOTISM (a mycotoxicosis)	*Claviceps purpurea*	Lameness is the first sign and occurs 2-6 weeks after ingestion of ergot alkaloids. All ages can be affected. There is tenderness and swelling in the fetlock and pastern joints, followed in about 1 week by loss of sensation and dry gangrene of the distal part of the limb	• History of pasture or foodstuffs contaminated by ergots • Clinical signs
FOOT ROT	*Fusobacterium necrophorum*, *Bacteroides melaninogenicus* and *Actinomyces pyogenes*	Infection is usually confined to one foot. There is acute lameness and paring of the horn of the sole liberates a foul-smelling exudate. Infection can spread to involve joints if not treated	• Clinical signs • Gram-stained smear from pus • Isolation of pathogens if necessary
JOINT-ILL	*Escherichia coli* *Staphylococcus aureus* Streptococci or others	Lodgement of pathogenic bacteria in joints following an omphalitis or a septicaemia. In the acute phase there is fever, severe lameness and swollen joints	• Clinical signs • Isolation of pathogen from aspirated joint fluid
LAMENESS (GENERALISED DISEASES) **Foot-and-mouth disease** **Vesicular stomatitis**	*Aphthovirus*, *Vesiculovirus*, *Pestivirus*	In foot-and-mouth disease and vesicular stomatitis, lameness is due to interdigital vesicular lesions, and all four feet are usually affected. Lameness occurs in some animals with mucosal disease and is due to laminitis, coronitis or erosive lesions of skin in the interdigital cleft. All four feet are usually affected	• Tests for detection of viral Ag, virus isolation, and demonstration of antibodies to each virus
MALIGNANT OEDEMA	*Clostridium septicum*, *C. sordellii* or *C. novyi* type A	Animals of all ages affected. If alive, there is fever and soft swelling around a wound that spreads to muscle masses. The swelling pits on pressure and if muscle is incised the tissue is dark. Exudate and gas are present	• Clinical signs • FA in muscle or bone marrow from a rib • Isolation if necessary

Table 168. Infectious diseases of cattle. *(continued)*

MUSCULOSKELETAL SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
MYCOPLASMAL ARTHRITIS	*Mycoplasma bovis*	Recognised most frequently in feed-lot cattle, 6-8 months of age. There is moderate fever, stiffness, lameness and progressive weight loss. Swelling of joints and distension of tendon sheaths occurs associated with fibrinous synovitis and synovial fluid effusions	• Clinical signs • Isolation of *M. bovis* from joint fluid (transport medium is required)
OSTEOMYELITIS	*Salmonella* spp., *Brucella abortus*, *Actinomyces bovis*	Vertebrae are often affected leading to pressure on the spinal cord or extension of infection to meninges. There is often ataxia and eventually hemiplegia or paraplegia depending on the site of the lesion. Lumpy jaw *(A. bovis)* is a specific disease affecting bone and soft tissue in the jaw region	• Clinical signs and X-ray examination • Pathology • Isolation of pathogen from CSF or lesion
TERMINAL DRY GANGRENE	*Salmonella dublin*	Ischaemic necrosis of tips of ears, tail and distal part of hind limbs can follow a few weeks after recovery from acute diarrhoeal disease. The condition characteristically occurs in young calves and is thought to be a localised form of disseminated intravascular coagulation	• History of previous illness • Differentiate from ergotism
TETANUS	*Clostridium tetani*	Tetanus may occur in cows following dystocia, calves can be infected via the umbilicus, castration or dehorning wounds, and occasionally all ages via deep wounds or injections. There is muscle stiffness, raised tail-head, bloat, tetanic spasms and opisthotonos	• History and clinical signs • Gram-stained smear of necrotic tissue deep in wound
THROMBOEMBOLIC MENINGOENCEPHALITIS	*Haemophilus somnus*	Bacterial colonisation of the meningeal vessels produces a thrombolic vasculitis leading to encephalitis and meningitis. There is fever, and with CNS involvement, motor and behavioural abnormalities develop such as stiffness, ataxia, stupor and opisthotonos	• Clinical signs • Pathology • Isolation of *H. somnus* from brain lesions

Table 168. Infectious diseases of cattle. *(continued)*

RESPIRATORY SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
CALF DIPHTHERIA	Trauma (coarse feed) and *Fusobacterium necrophorum*	Necrotic lesions in oral, pharyngeal or laryngeal mucosa. Fever, anorexia and salivation are seen and the condition can lead to pneumonia	• Gram-stained smear on scrapings from lesions • Isolation (anaerobic)
CONTAGIOUS BOVINE PLEUROPNEUMONIA (CBPP)	*Mycoplasma mycoides* ss. *mycoides* (small colony type)	**Acute**: 'Marbling' of lungs and a large volume of fluid in thorax. **Chronic**: Necrosis and walling-off of portion of lungs. These animals can be latent carriers or 'lungers' for up to 3 years. In time the lesion may break down and mycoplasmas are shed.	• Gross pathology and histopathology • Isolation from lung or pleural fluid • Serology: CFT and AGID
ENZOOTIC PNEUMONIA	Complex involves some or all of the following pathogens: Parainfluenzavirus 3 (PI3); bovine respiratory syncytial virus; bovine viral diarrhoea (BVD) virus; infectious bovine rhinotracheitis (IBR); *Mycoplasma bovis; M. dispar; Pasteurella haemolytica; Pasteurella multocida* and *Haemophilus somnus*	Predisposing causes are important in this disease complex and morbidity can reach 100 per cent. Mainly a problem of intensively reared calves 2-6 months of age. There is fever, depression, increased respiratory rate and coughing. Gradual recovery unless a severe bacterial pneumonia develops. Lesions usually in anteroventral portion of lungs	• Isolation and identification of pathogen(s). Pasteurellae are isolated in later stages, by then the isolation of viruses and mycoplasmas is difficult • Serology: for viruses
'FARMER'S LUNG' IN CATTLE	Sensitisation to spores of thermophilic actinomycetes such as: *Micropolyspora faeni*	Immediate type hypersensitivity. Condition due to mouldy hay fed in an enclosed area. Clinical signs of respiratory distress are seen at the end of winter. A few acute cases occur but the condition is usually chronic	• Usually only 1 or 2 acute cases which occur in cattle over 5 years old • Serology: AGID antibodies to *M. faeni*
HAEMORRHAGIC SEPTICAEMIA	*Pasteurella multocida* serotype B:2 (Asia) or E:2 (Africa)	An acute pasteurellosis characterized by a rapid course, oedematous swelling in the head-throat-brisket area, respiratory distress, swollen and haemorrhagic lymph nodes and subserous petechiation. Cattle and water buffaloes are susceptible	• History of endemic area • Isolation of pathogen from heart-blood, liver, spleen or lymph nodes • Serotype identification

Table 168. Infectious diseases of cattle. *(continued)*

RESPIRATORY SYSTEM: cattle

Disease	Agent(s)	Comments	Diagnosis
INFECTIOUS BOVINE RHINOTRACHEITIS (IBR)	Bovine herpesvirus 1	Most common in young cattle under stress such as in feedlots. Nasal discharge, 'red nose', ulcers of mucous membranes in nasal passages, conjunctivitis, mouth breathing. Recovery 4-5 days without secondary bacterial invasion. Abortions 3-4 weeks after respiratory disease in pregnant adults	• Virus isolation or FA: nasal and eye swabs or nasopharyngeal aspirate early in disease • FA: frozen sections from aborted foetuses • Histopathology (IBs transitory, occasionally seen) • Serology: VN or ELISA
INHALATION PNEUMONIA	Anaerobes from rumen.	Following milk fever or general anaesthesia	• History • PM findings
LUNG ABSCESSES	*Actinomyces pyogenes*	Occurs alone or as a complication of other respiratory diseases, especially in pasteurellosis of young cattle	• Direct microscopy • Isolation and identification
MYCOTIC PNEUMONIA	*Aspergillus fumigatus* or *Mortierella wolfii*	Usually chronic except for peracute and fatal *M. wolfii* pneumonia that occurs within 48 hours of abortion . The lungs are wet, oedematous with a large volume of fluid in thorax	• Histopathology • Isolation and identification of pathogenic fungi
SHIPPING FEVER (Bovine pneumonic pasteurellosis)	Parainfluenzavirus 3 (PI3); *Pasteurella haemolytica*; *Pasteurella multocida*; *Haemophilus somnus*	Aetiology involves stress + virus + bacteria. All ages can be affected and deaths are usually due to an overwhelming *P. haemolytica* infection. The disease varies from mild pneumonia to a fulminating bronchopneumonia	• Isolation and identification of bacteria • Serology for PI3: IFA, HAI, VN (rising Ab titre)
TUBERCULOSIS (BOVINE)	*Mycobacterium bovis*	Tubercles in lymph nodes, lungs and pleural cavity. 'Open cases' can create an aerosol of *M. bovis*	• Tuberculin test • ZN-smears on tissue • Isolation and identification of organism • Blood-based diagnostic tests currently being evaluated.

SKIN: cattle

ACTINOBACILLOSIS	*Actinobacillus lignieresii*	Classical disease is a granulomatous infection of the tongue: wooden or timber tongue. But the pathogen can cause granulomatous lesions of skin with purulent exudates from fistulae anywhere on body including the jaw area. Bone not involved and prognosis is usually good	• Microscopy of crushed granules from pus: Gram -ve rods • Culture: aerobic

Table 168. Infectious diseases of cattle. *(continued)*

SKIN: cattle

Disease	Agent(s)	Comments	Diagnosis
BACTERIAL ABSCESSES	*Staphylococcus aureus,* Streptococci, or *Actinomyces pyogenes*	Infection usually via abrasions and other trauma such as injections by a non-aseptic technique	• Gram-stained smear from pus. • Culture for pathogen.
BOVINE PAPILLOMATOSIS	Bovine Papillomaviruses types 1-6 (*Papovaviridae*)	Cutaneous warts (types 1, 2 and 3), teat warts (types 5 and 6) and type 4 occurs in bladder or intestines. Affects all ages but highest incidence in calves and yearlings. Type 1 and 2 warts range from small nodules to cauliflower-like growths. Most common on head and neck. Self-limiting condition	• Clinical signs • EM • Histopathology
BOVINE VIRAL DIARRHOEA IN DAM **(congenital alopecia)**	*Pestivirus* (*Flaviviridae*)	Infection of dam during mid-gestation. The dam may or may not show clinical signs. Congenital defects in calves infected with the virus are various, including cerebellar hypoplasia, cataracts and alopecia. Such calves if born alive may be immunotolerant and persistently infected (V+ve/Ab-ve)	• FA on buffy coat smear for virus, frozen sections • Serology: VN or ELISA • Virus isolation
DERMATOPHILOSIS **(Streptothricosis, Mycotic dermatitis)**	*Dermatophilus congolensis*	Reservoir often small foci on carrier animal. Condition most common in young animals. Predisposing causes include: wet conditions (lesions along backline), abrasions from vegetation (muzzle, face and limbs) and tick infestation (tick predilection sites). Exudative dermatitis with extensive scab formation occurs and scab comes away with tufts of hair leaving a raw bleeding surface	• Gram or Giemsa-stained smears from scab. Filamentous and branching with zoospores at least two across; 'tram-track' appearance • Culture: 10 per cent CO_2
FACIAL ECZEMA	Sporidesmin in spores of *Pithomyces chartarum* (mycotoxin)	Mycotoxicosis of grazing cattle and sheep. Fungus grows in pasture litter under moist, warm conditions. Liver damage and biliary obstruction occurs that restricts excretion of bile pigments and jaundice can occur. Failure to excrete phylloerythrin leads to photosensitization with lesions in non-pigmented skin including the udder and ears	• Spore counts on pasture • Gross pathology: liver • Histopathology
LUMPY JAW	*Actinomyces bovis*	Granulomatous lesions in jaw region with abscesses and fistulous tracts exuding pus. Bone is attacked and once rarefying osteitis becomes extensive the prognosis is poor	• Microscopy of crushed sulphur granules from pus: Gram +ve filaments • Culture: anaerobic

Table 168. Infectious diseases of cattle. *(continued)*

SKIN: cattle

Disease	Agent(s)	Comments	Diagnosis
LUMPY SKIN DISEASE	*Orthopoxvirus* (Neethling virus) (*Poxviridae*)	Limited to Africa. Nodules in skin all over body with general lymphadenitis, oedema of the limbs, nasal discharge and internal organs including lungs are involved. There is fever and anorexia. Mortality only 1-2 per cent but animals remain debilitated for long periods. Nodules ulcerate and heal slowly leaving scars and alopecia	• Histopathology on biopsy: IBs • EM: demonstration of poxvirus particles • Isolation: CAM or TC
PSEUDOLUMPY SKIN DISEASE	Bovine herpesvirus 2 (Allerton virus)	This syndrome seen mainly in Africa but can occur elsewhere. The virus also causes bovine ulcerative mammillitis in Europe and North America. Pseudolumpy skin disease is a comparatively mild disease with fever and skin nodules over the body. The nodules undergo necrosis with central depression but no scar. Mortality very low	• EM • Histopathology on biopsy: IN inclusion bodies • Virus isolation • Serology: VN, CFT, AGID, IFA
PSEUDORABIES ('Mad itch')	Porcine herpesvirus 1	Reservoir is usually pigs with Aujeszky's disease. Rats may take virus from farm to farm. Infection mainly by ingestion and less commonly by inhalation or via wounds (pig bites). Dominant sign is an intense pruritis; mainly flanks and hind limbs. Incessant licking, biting and rubbing so infected areas become abraded. Progressive involvement of CNS with frenzy and bellowing but not aggression. Death may occur within a few hours to a maximum of 6 days after first signs	• History of association with pigs • Clinical signs • Diagnosis of Aujeszky's disease in pigs • FA: frozen sections • Virus isolation
RINGWORM	*Trichophyton verrucosum*	Usually seen in calves or yearlings. The lesions are most common on the face, around the eyes and on the neck. They are circular and later develop a grey - white crust. Self-limiting disease	• Hairs in 10-20 per cent KOH • Culture at 27°C and 37°C, for 6 weeks
SKIN TUBERCULOSIS	Acid-fast bacterium (unnamed)	Chronic indurative nodules associated with the presence of acid-fast bacteria. Usually occurs on the lower limbs. The affected animals often react to the tuberculin test. The acid-fast bacteria have not yet been cultured	• Clinical signs • ZN-stained smears
VESICULAR DISEASES	Foot-and-mouth disease: (*Aphthovirus*, *Picornaviridae*) Vesicular stomatitis: (*Vesiculovirus* , *Rhabdoviridae*)	Vesicular lesions on muzzles, buccal cavity, interdigital cleft and on teats of milking animals	• Viral Ag detection : CFT, ELISA (vesicle fluid or epithelial material) • Virus isolation • Serology: VN, ELISA, CFT

Table 169. Infectious diseases of sheep (goats).

BUCCAL CAVITY: sheep (goats)

Disease	Agent(s)	Comments	Diagnosis
BLUETONGUE	*Orbivirus (Reoviridae)*	The highest losses are in growing lambs. There is depression, fever, oedema of lips, tongue and throat. The buccal mucosa is erythematous or cyanotic and erosions appear on the dental pad, tongue, gums and lips. The muzzle is usually encrusted. Stiffness and lameness occur	• History of endemic area • Virus isolation • Serology: VN, ELISA, AGID, modified CFT
CONTAGIOUS PUSTULAR DERMATITIS (ORF)	*Parapoxvirus (Poxviridae)*	Scab formation is usually restricted to the lips and nostrils but, as there is no colostral immunity, the disease can be severe in young lambs and kids. With stiff, sensitive lips and sometimes lesions in the buccal cavity, the young animals cannot suck or graze	• Clinical signs • EM for parapoxvirus particles • Histopathology on biopsy. • Virus isolation
FOOT-AND-MOUTH DISEASE	*Aphthovirus (Picornaviridae)*	Lameness, usually in all four feet due to vesicular lesions of the interdigital clefts, is the constant finding in sheep. Occasionally vesicular lesions occur in the buccal cavity	• Clinical signs • Viral Ag: CFT, ELISA on vesicular fluid • Virus isolation • Serology: ELISA, CFT, VN
PESTE DES PETITS RUMINANTS	*Morbillivirus (Paramyxoviridae)*	Highly contagious, systemic disease of sheep and goats (West Africa), although many infections are subclinical. Signs include fever, anorexia, necrotic stomatitis with gingivitis and diarrhoea. Mortality in goats can reach 95 per cent, sheep slightly less susceptible	• Gross and histopathology • AGID and CFT on lymph node biopsies for Ag • Virus isolation: it is distinguished from rinderpest virus using cross-neutralisation tests • Serology: VN, HAI, AGID
RINDERPEST	*Morbillivirus (Paramyxoviridae)*	Sheep and goats are susceptible to infection but disease is usually mild. A few large and serious outbreaks have been reported with signs similar to those in cattle: severe diarrhoea and shallow erosions of lips, dental pads and gums	• AGID and CFT on lymph node biopsies for Ag • Histopathology • Virus isolation • Serology: CFT, ELISA, VN, HAI
SHEEP POX (GOAT POX)	*Capripox (Poxviridae)*	The disease affects all ages. There is fever, rhinitis, anorexia and generalized pox eruptions on skin and mucosa of buccal cavity and pharynx within 1-2 days of first signs. Gaseous nodules and catarrhal pneumonia occur in the lungs. Mortality varies from 5-50 per cent	• History of endemic area and clinical signs • EM • AGID for viral Ag • Histopathology • Virus isolation

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Cover figure: *Nocardia asteroides* complex in an expectorated sputum specimen stained with fluorescein. The photograph was taken with a Zeiss fluorescent microscope at a magnification of ×1,000. (*Courtesy of Patrick R. Murray.*)

Escherichia, Salmonella, Shigella, and *Yersinia*

LARRY D. GRAY

33

Salmonella, *Shigella*, and *Yersinia* spp. and certain strains of *Escherichia coli* are enteric pathogens capable of causing severe gastroenteritis and life-threatening systemic illnesses (17).

ESCHERICHIA SPP.

E. coli is the most common bacterium isolated in clinical microbiology laboratories, the most prevalent facultative gram-negative rod in feces, the most common cause of urinary tract infection, and a common cause of both intestinal and extraintestinal infections (see chapter 32 of this Manual) (1, 2, 13, 17, 23, 27, 37).

E. coli Strains That Cause Diarrhea

E. coli is part of the healthy bowel fecal flora of both humans and lower animals; however, some strains of *E. coli* can cause severe and life-threatening diarrhea. Five distinct groups of *E. coli* cause gastrointestinal illnesses ranging from mild diarrhea to choleralike diarrhea to potentially fatal complications such as hemolytic uremic syndrome (HUS) (Table 1) (1, 2, 13, 30, 37). Enterotoxigenic *E. coli* (ETEC) produces cholera toxin-like enterotoxins that elicit profuse watery diarrhea. Enteropathogenic *E. coli* (EPEC) causes infantile diarrhea. Enteroinvasive *E. coli* (EIEC) invades intestinal epithelium and produces dysentery similar to that caused by *Shigella* spp. Enterohemorrhagic *E. coli* (EHEC) is a defined subset of Shiga-like (vero) toxin-producing *E. coli*. At least one serotype (O157:H7) of EHEC can cause hemorrhagic colitis and HUS. Enteroaggregative *E. coli* (EAggEC) is the most recently described diarrheagenic *E. coli*.

Strains of *E. coli* that cause intestinal infections resemble healthy bowel strains on common plating media and in biochemical tests (17). Unlike *Salmonella* and *Shigella* spp., pathogenic strains of *E. coli* can be lactose positive or negative. After they are subcultured, these strains can be serotyped (e.g., *E. coli* O157:H7) or tested in specific virulence assays.

ETEC

Characteristics

ETEC causes dehydrating infantile diarrhea in developing countries and traveler's diarrhea, but it is rare in the United States. ETEC can produce nausea, abdominal cramps, low fever, and a sudden-onset, profuse, watery diarrhea that is not unlike a mild case of cholera. Traveler's diarrhea caused by ETEC can be severe but is rarely fatal. ETEC produces plasmid-mediated, cell-associated heat-stable enterotoxins (ST) and heat-labile enterotoxins (LT) (13, 37).

Detection of ETEC Enterotoxins

The assays for LT and ST are relatively complicated, often involve biological systems or cell culture, and are performed primarily in reference or research laboratories. However, commercial products for the detection of ETEC LT and ST are available (Unipath [Oxoid], Ogdensburg, N.Y.). Cell culture assays for LT are described in chapter 14 of this Manual.

EPEC

Characteristics

EPEC causes enteric diseases (particularly in small children) characterized by fever, vomiting, and prominent and watery diarrhea, usually with mucus but not blood (1, 37). The first *E. coli* isolates shown to be enteric pathogens were described as such in 1955 and were named EPEC at that time. Since 1950, O55, O111, and other "enteropathogenic serotypes" have been shown to be important causes of worldwide infantile diarrhea.

Detection

EPEC can be isolated on routine enteric media. Polyvalent antisera for detecting EPEC O-antigen groups are still commercially available. In developed countries, these antisera probably do not have a place in routine laboratory procedures, but they can be used in neonatal outbreaks and cases of severe or chronic diarrhea. Five to 10 lactose-positive colonies of *E. coli* should be picked and examined for agglutination in specific antiserum. Strains that agglutinate in the serologic tests must be confirmed by titration according to the manufacturer's instructions (to eliminate cross-reactions) and should be submitted to a reference laboratory for confirmation and determination of H antigen (i.e., complete O:H typing). EPEC also has been detected by enzyme-linked immunosorbent assays (ELISA) and cell culture assays (37).

TABLE 1 Properties of *E. coli* strains that cause enteric infections

Strain	Pathogenic mechanisms	Enteric infection(s)	Common clinical presentations	Common age group	Common risk factor
ETEC	LT and ST	Diarrhea; traveler's diarrhea	Profuse watery diarrhea, cramps, nausea, dehydration	Adults, children	Foreign travel (usually Mexico)
EPEC	Adherence factor; attachment to and effacement of intestinal epithelium	Acute diarrhea	Watery diarrhea, fever, vomiting, mucus in stool	Children <2 yr old, adults	Age <2 yr
EIEC	Invasion and destruction of intestinal mucosal epithelium	Dysentery similar to *Shigella* dysentery	Dysentery; scant stool; blood, mucus, and leukocytes in stool; fever; cramps	Adults	Foreign travel (usually Mexico)
EHEC	Shiga-like toxins	Diarrhea; hemorrhagic colitis	Diarrhea (no leukocytes); abdominal cramps; blood in stool; fever, HUS, and TTP[a] may or may not be present	Children, elderly	Consumption of undercooked ground beef
EAggEC	Unknown	Chronic and acute diarrheas	Watery diarrhea, vomiting	All ages	Unknown

[a]TTP, thrombotic thrombocytopenic purpura.

EIEC

Characteristics

Like *Shigella* spp. and unlike most *E. coli* strains associated with enteritis, EIEC invades the colonic mucosa, multiplies within mucosal epithelial cells, and disrupts the epithelial cells. Also like *Shigella* spp., EIEC produces enteritis characterized by fever, abdominal cramps, watery diarrhea, or typical bacillary dysentery with leukocytes (often excreted in sheets), blood, and mucus. In most studies, EIEC appears to be relatively rare in both developing and developed countries (17).

Detection

Like *Shigella* spp., most EIEC strains are nonmotile and non- or late lactose fermenters. EIEC can be isolated on routine enteric media and can be identified by the following: Sereny test, O:H serogrouping, ELISA, or invasiveness into HEp-2 or HeLa cells. All EIEC strains are lysine decarboxylase negative, and almost all are nonmotile. The most common serotypes of EIEC are biochemically lactose positive; serotypes O152 and O124 are lactose negative. Antigenically, EIEC can cross-react with antisera to *Shigella* spp.

EHEC

Characteristics

EHEC strains are water borne and food borne. EHEC is ingested most commonly with undercooked ground beef (10, 22, 28). Beef usually is contaminated at slaughterhouses, where it contacts bovine feces. Person-to-person transmission occurs by the oral-fecal route, and appropriate infection precautions should be observed. EHEC produces two distinct lysogenic bacteriophage-encoded toxins that inhibit protein synthesis and are active against Vero and HeLa cells: Shiga-like toxin 1 (verotoxin 1) and Shiga-like toxin 2 (verotoxin 2) (22, 27). Unfortunately, simple methods and commercially available products for detecting strains that produce Shiga-like toxins do not exist; however, tests to detect strains that produce Shiga-like toxins can be performed by some reference laboratories. The Vero cell assay for Shiga-like toxins is described in chapter 14 of this Manual.

There are >50 serotypes of EHEC (17, 22). However, *E. coli* O157:H7 is the prototype EHEC. It is by far the most studied and publicized serotype, the only serotype that can be easily isolated and identified in most clinical microbiology laboratories, and the only EHEC that is a public health problem. Almost all published information regarding EHEC applies only to *E. coli* serotype O157:H7. In most geographic areas, *E. coli* O157:H7 is the most frequently isolated EHEC. Non-O157 EHEC strains are not common causes of diarrhea and HUS and have not been shown to cause bloody diarrhea or outbreaks of diarrhea in the United States or Canada (22, 28).

In many parts of North America, *E. coli* O157:H7 often is the second or third most commonly isolated enteric bacterial pathogen (usually isolated more often than *Shigella* and *Yersinia* spp.). It is usually the most common bacterial pathogen isolated from bloody stools and has been isolated from as many as 40% of all bloody stools (22). The occurrence of *E. coli* O157:H7 infections is greatest in June, July, and August in temperate climates (22).

Clinical Manifestations

After an incubation period of 3 to 5 days (range, 1 to 8 days), *E. coli* O157:H7 can cause an asymptomatic infec-

tion, mild diarrhea, or a diarrheal illness that is characterized by nonbloody (progressing to bloody) diarrhea and abdominal cramps (together known as hemorrhagic colitis), few leukocytes in stools, and lack of significant fever (10, 22). In adults, the illness usually is self-limited, lasts 5 to 8 days, and resolves without sequelae. Of patients with *E. coli* O157:H7 diarrhea, 2 to 7% develop HUS, the most important complication of EHEC infection. HUS is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure (27). *E. coli* O157:H7 is the leading cause of acute renal failure in children less than 4 years old (27). Persons who develop HUS have mortality rates of 3 to 10% and severe or chronic renal, cardiac, and neurologic complication rates of 4 to 30% (10, 27).

Detection and Identification

In 1993, the Centers for Disease Control and Prevention (CDC) recommended that all laboratories culture routinely for *E. coli* O157:H7 (10, 28). Ideally, each laboratory should culture for *E. coli* O157:H7 routinely for a few months to a year to determine the prevalence of the bacterium and then decide whether routine culture for *E. coli* O157:H7 is cost-effective. This suggestion will not be practical for many laboratories. Alternatively, other surveillance plans can be implemented: only patients with bloody stools could be cultured for *E. coli* O157:H7 routinely, all stools could be cultured routinely for *E. coli* O157:H7 but only during the months with the highest incidence of the disease, etc. (28).

Stools from adults and children should be cultured within 7 and 30 days, respectively, of the onset of illness. Ideally, stool from any patient who reports having bloody diarrhea should be cultured for *E. coli* O157:H7, because stool from a patient who has had bloody diarrhea does not always appear bloody in the laboratory.

Approximately 80% of most healthy bowel fecal flora *E. coli* ferment D-sorbitol in ≤24 h (28). On the other hand, virtually all *E. coli* O157:H7 do not ferment (or ferment very slowly) D-sorbitol, a characteristic used to differentiate *E. coli* O157:H7 on MacConkey-sorbitol agar medium (SMAC). Therefore, *E. coli* colonies that do not ferment D-sorbitol (colorless colonies after 24 to 48 h at 35 to 37°C) can be screened directly from SMAC or after subculture with one of two latex agglutination tests for *E. coli* O157 antigen (Unipath [Oxoid]; Pro-Lab Diagnostics, Round Rock, Tex.) (31, 42). If SMAC is used for primary culture and selection, only three colonies need to be screened for the presence of O157 antigen.

Unlike 92 to 96% of all *E. coli*, most Shiga-like-toxin-producing isolates of *E. coli* O157:H7 do not produce β-D-glucuronidase and cannot cleave 4-methylumbelliferyl-β-D-glucuronide (MUG) to an end product that is visible under 366-nm UV light; therefore, they are MUG negative (28, 41). A MUG test is commercially available (Remel, Lenexa, Kans.).

Before any report is issued, the isolate must be proven to be *E. coli* by standard biochemical tests. Sorbitol-negative *E. coli* O157:H7 isolates do not need to be tested for toxin production, because virtually all of these isolates produce toxin.

Susceptibility to Antimicrobial Agents

There is no evidence that use of antimicrobial agents to treat *E. coli* O157:H7 disease changes the course of the disease or is beneficial in any way (21, 27).

EAggEC

The term enteroadherent *E. coli* refers to *E. coli* strains that do not produce LT or ST; are not invasive; usually are not grouped according to O:H serotypes as ETEC, EPEC, EIEC, or EHEC; and adhere to HEp-2 and HeLa cells in characteristic patterns (35, 37).

EAggEC is the best-studied enteroadherent *E. coli*. EAggEC has been associated with chronic diarrhea in many parts of the world. In children, EAggEC produces intestinal illness characterized by watery diarrhea, vomiting, dehydration, and, occasionally, abdominal pains, fever, and bloody stools.

Detection

The diagnosis of EAggEC-associated diarrhea can be accomplished only by a liquid-culture clump aggregation test, a test for adherence to cells, or a DNA probe test (4, 5, 35).

Species Other Than *E. coli*

In addition to *E. coli*, the genus *Escherichia* includes *Escherichia hermanii* (former CDC enteric group 11), *Escherichia vulneris* (former CDC enteric group 1), *Escherichia fergusonii* (former CDC enteric group 10), and *Escherichia blattae*. Although uncommon, these species occasionally are isolated from human specimens and appear to be potential pathogens (16). *Escherichia adecarboxylata/agglomerans* has been assigned to a new genus (*Leclercia adecarboxylata*).

SALMONELLA SPP.

Salmonella spp. have been isolated from humans and almost all animals throughout the world. Some serotypes of *Salmonella* are virtually species specific. For example, humans are the only known natural reservoir for serotype *Salmonella typhi* and (for all practical purposes) serotypes *Salmonella paratyphi* A, B, and C.

Salmonella spp. cause many types of infections, from mild self-limiting gastroenteritis to life-threatening typhoid fever. The most common form of *Salmonella* disease is self-limiting gastroenteritis with fever lasting less than 2 days and diarrhea lasting less than 7 days. Typhoid fever, the best-studied enteric fever, is characterized by fever, headache, diarrhea, and abdominal pain and can produce fatal respiratory, hepatic, spleen, and/or neurologic damage. Bacteremia, meningitis, respiratory disease, cardiac disease, osteomyelitis, and other local infections caused by *Salmonella* spp. have also been reported (25).

S. typhi and *S. paratyphi* A and B cause gastroenteritis, bacteremia, and enteric fever; *Salmonella choleraesuis* causes gastroenteritis and enteric fever, especially in children; and *Salmonella typhimurium* is the most frequently isolated serotype in the United States. The first four serotypes are not common in the United States. Serotypes other than the first four are most likely to cause uncomplicated gastroenteritis.

Nomenclature and Classification

Nomenclature and classification of these bacteria have changed many times and still are not stabilized (15, 17, 18). The genera *Salmonella* and (the former) *Arizona* are so closely related in evolutionary lines and degree of DNA homology that they should be considered a single genus: *Salmonella* (18). Most experts agree that there are seven distinct subgroups of *Salmonella*, each with its own phenotypic characteristics and its own historical progression of

nomenclature. See the previous edition of this Manual (10) for a useful and practical tabular presentation of the characteristics of the seven subgroups of *Salmonella*. Most salmonellae (>99%) isolated in clinical microbiology laboratories belong to subgroup 1.

Serotypes

The members of each of the seven subgroups of *Salmonella* can be serotyped according to somatic O, surface Vi, phase 1 flagellar, and phase 2 flagellar antigens (17). The detailed antigenic description of each serotype is always given in a notation in which major antigenic groups are separated by colons (O antigens:Vi antigen:phase 1 antigens:phase 2 antigens). For simplicity and convenience, each of the most important serotypes usually is named for the location where it was first isolated or for something else with which it is associated (i.e., each serotype is treated and named as if it were a species). For example, *Salmonella* subgroup 1 (choleraesuis) serotype 1,4,5,12:i:1,2 can be called "*Salmonella*, subgroup 1, serotype typhimurium" or "*Salmonella*, serotype typhimurium"; however, it is usually called simply *S. typhimurium*. Experts at the CDC have stated that this simple nomenclature is widely accepted, practical, and clinically informative (18).

Most laboratories use commercially available polyvalent antisera to determine the O-antigen groups of isolates of *Salmonella* (serogroup A, B, C1, C2, D, etc.) and to provide rapid and preliminary identification (e.g., *Salmonella* serogroup B) to physicians. Preliminary serogrouping is important, because certain results of serogrouping can suggest that an isolate might be one of the five important serotypes. Specifically, the well-recognized isolates *S. paratyphi* A, *S. typhimurium*, *S. paratyphi* B, *S. choleraesuis*, and *S. typhi* are members of serogroups A, B, B, C, and D, respectively. Most state laboratories perform complete O:H serotyping of *Salmonella* isolates. If an isolate fails to agglutinate in polyvalent antiserum, a glass test tube containing a very heavy suspension (in saline) should be placed into boiling water for 15 to 30 min (longer times can be required) and cooled, and the suspension should be retested with the antiserum. Boiling destroys the surface Vi capsular antigen that can block agglutination.

Three commercially available latex agglutination tests can be used for the rapid detection of *Salmonella* and *Shigella* spp. after growth on solid media and in broth (Wellcolex Colour *Salmonella* and Wellcolex Colour *Shigella*, Murex, Kent, England; Bactigen *Salmonella-Shigella* Test, Wampole Laboratories, Cranbury, N.J.) (8, 19, 24, 33, 38).

Isolation

Routine selective and differential enteric agar media usually are sufficient for isolating *Salmonella* and *Shigella* spp. (12). Both a differential enteric agar medium (MacConkey or eosin-methylene blue) and a moderately selective agar medium (*Salmonella-Shigella*, xylose-lysine-deoxycholate, or Hektoen) should be used. Highly selective enteric agar media (brilliant green and bismuth sulfate) can be reserved for use only during outbreaks. Brilliant green agar is especially good for isolating *Salmonella* spp. (except *S. typhi* and *S. paratyphi*), and bismuth sulfate agar is effective for isolating *S. typhi*.

An enrichment broth (selenite or gram negative [GN]) can be added to the primary media to facilitate the recovery of small numbers of *Salmonella* and *Shigella* spp. However, enrichment broths are not cost-effective enough for routine use except during outbreaks, when screening for carriers, and in other clinically warranted situations.

Identification

Suspicious colonies of *Salmonella* spp. can be identified by manual or commercial biochemical methods. A less expensive (but usually 1-day-longer) screening test for *Salmonella* spp. uses triple sugar iron (TSI) agar and lysine iron agar. The typical biochemical pattern on TSI is alkaline/acid, gas positive, and H_2S positive. Some strains are lactose positive and acid/acid, but these strains usually are H_2S negative. The typical pattern on lysine iron agar is alkaline/alkaline and H_2S positive; however, lysine- and H_2S-negative strains exist. *S. paratyphi* A is both lysine and H_2S negative. To reduce laboratory expenses without decreasing the quality of clinically relevant results, some laboratories no longer serogroup *Salmonella* and *Shigella* spp. but instead send isolates to reference laboratories or to local or state public health laboratories for serogrouping. Isolates that give appropriate preliminary biochemical reactions should be serogrouped with commercially available polyvalent antisera, and a preliminary report should be issued. Subsequently, the isolate must be fully identified biochemically and fully serotyped by a reference laboratory before a final report is issued.

The 4-methylumbelliferyl caprilate fluorescence disk test (Remel) for detecting C8-esterase can be used to screen stool isolates for *Salmonella* spp. False positives can be caused by other members of the family *Enterobacteriaceae* (3, 34, 36, 39).

Serodiagnosis

The Widal test for antibodies to the O antigens of *Salmonella* serotypes most likely to cause typhoid fever, usually *S. typhi* and *S. paratyphi* A and B, can be useful in helping diagnose typhoid fever when other methods have failed (40).

SHIGELLA SPP.

Shigella spp. cause classic bacillary dysentery (shigellosis), a descending intestinal illness characterized by abdominal pain, fever, large volumes of watery stools, and, 1 to 2 days later, smaller volumes of stools that often contain much blood and mucus and many leukocytes. The pathogenicity of shigellosis involves invasion and inflammation of the colonic epithelium, destruction of the superficial mucosa, sloughing of the mucosa, and production of mucosal ulcers. *Shigella* spp. rarely invade beyond the mucosa; recovery of the bacterium in blood is rare. *Shigella dysenteriae* can cause a particularly severe form of dysentery that has been reported to have fatality rates of up to 20%. Infection with *Shigella* spp. other than *S. dysenteriae* usually is self-limited and rarely fatal except in the elderly and in undernourished children.

Most cases of shigellosis are individual cases and are due to person-to-person transmission. When associated with outbreaks, the disease usually is transmitted by contaminated food and/or water.

The four named species-serogroups of *Shigella* are *S. dysenteriae* (serogroup A), *Shigella flexneri* (serogroup B), *Shigella boydii* (serogroup C), and *Shigella sonnei* (serogroup D). Serotyping *Shigella* spp. beyond the serogroup level is usually done only by reference laboratories and is extremely useful and even imperative in epidemiologic investigations.

Shigella spp. first showed resistance to sulfonamides in

the 1950s and have developed multiple resistances since that time. *Shigella* isolates became resistant to tetracycline, then to ampicillin in the 1970s and 1980s, and recently to trimethoprim-sulfamethoxazole. This development of multiple resistance is especially prominent and problematic outside the United States. However, multiply resistant *Shigella* isolates are becoming increasingly common in the United States.

Specimen, Media, and Identification

In order of decreasing productivity, rectal swab specimens, stool, and anal swab specimens are the specimens of choice (14). Streaks and collections of blood, mucus, and pus in stool specimens are extremely productive and should be cultured.

For optimal chances of isolating *Shigella* spp., a differential enteric agar medium and a moderately selective agar medium should be used (20). Xylose-lysine-deoxycholate medium is especially good for isolating *Shigella* spp.

Suspect colonies can be identified directly in many commercial identification systems. Alternatively, suspect colonies can be screened for *Shigella* spp. by subculturing the colonies onto TSI or Kligler iron agar (41). Isolates that are alkaline/acid on TSI or Kligler iron agar, H_2S negative, and gas negative can be further identified to genus and species. *Shigella* spp. other than *S. sonnei* should be sent to a state or reference laboratory for serotyping.

Occasionally, biochemically identified isolates of *Shigella* will not agglutinate in *Shigella* antisera. Suspensions of these isolates should be heated in a water bath at 100°C for 15 to 30 min and retested for agglutination. Such isolates could be EIEC.

Differentiation of *Shigella* spp. and *E. coli*

Taxonomically, *Shigella* spp. and *E. coli* are essentially the same genus and species. Their DNA relatedness is very high, they are often difficult to differentiate biochemically, and they cross-react serologically (see chapter 32 of this Manual). However, they have remained separate species for clinical reasons. The following are suggested guidelines for identifying *Shigella* spp. (17).

1. Isolates are always nonmotile and lysine negative.
2. With the exception of a few strains of a few serotypes (*S. flexneri* 6, *S. boydii* 13 and 14, and *S. dysenteriae* 3), gas is not produced during carbohydrate fermentation.
3. Isolates that ferment mucate or are alkaline on acetate or Christensen citrate agar are likely to be *E. coli*.
4. *S. boydii* and *S. dysenteriae* are very rare in the United States, and isolates identified as one of these should be retested or confirmed in some way before a final report is issued.

YERSINIA SPP.

All 11 species of *Yersinia* are well established members of the family *Enterobacteriaceae*, and all have been isolated from clinical specimens. At least three species are unquestionably human pathogens: *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*. Yersinioses are zoonotic infections that usually affect rodents, small animals, and birds; humans are accidental hosts. Both *Y. pestis* and *Y. pseudotuberculosis* are rarely isolated in the United States.

Y. pestis

Y. pestis causes both urban and sylvatic plague. Rats are the natural reservoirs for *Y. pestis* in urban plague (the devastating "city" disease of the Middle Ages), and small animals such as ground squirrels, field and wood rats, rabbits, and domestic cats are the natural reservoirs in sylvatic plague (the "country" plague as it exists today in many countries, including the United States). Humans become infected with the bacterium when they are bitten by fleas of natural reservoirs, when they handle infected animals or tissues, and, much less commonly, when they inhale aerosolized *Y. pestis* generated by a person with pneumonic plague (12). The last reported case of human-to-human transmission of plague in the United States occurred in 1924.

Infections due to *Y. pestis* are rare in the United States; most (89%) of the cases have been reported from New Mexico, Arizona, California, and Colorado (12). From 1970 to 1991, there were 295 reported cases of indigenous plague in the United States (high year, 1983 [40 cases]).

Y. pestis is not a fastidious bacterium; it grows well on blood agar and many other enteric media. However, after 24 h on blood agar and many enteric agars, the colonies are only pinpoint, much smaller than those of other *Enterobacteriaceae*. After 48 h, the colonies are 1 to 1.5 mm in diameter and gray-white, and they can appear slightly mucoid.

Y. pestis is inactive in routine biochemical tests but has a typical pattern that closely resembles that of *Y. pseudotuberculoisis* (see chapter 32 of this Manual). Broth cultures of *Y. pestis* have a characteristic "stalactite pattern" in which clumps of cells adhere to the side of the tube and settle to the bottom if the tube is disturbed. Many commercial identification systems do not include *Y. pestis* in their data banks; therefore, *Y. pestis* must be suspected from its appearance on primary media and from clinical and epidemiologic information. Isolates suspected of being *Y. pestis* should be reported immediately by telephone to the state health department, which will request that the isolate be sent to a specialized reference laboratory. In the United States, isolates should be sent to Plague Section, Bacterial Zoonoses Branch, Centers for Disease Control and Prevention, P.O. Box 2087, Fort Collins, CO 80522; telephone, (303) 221-6450.

Y. enterocolitica

In the last several years, *Y. enterocolitica* has been isolated from many kinds of clinical and nonclinical specimens in many countries, particularly in Europe, Scandinavia, and Canada. Serotype O:3 is reported to be an increasingly significant enteric pathogen in the United States, especially in California, New York City, and some pediatric populations (6, 7, 29, 32). The natural reservoirs of the bacterium are many kinds of animals, especially pigs, rodents, livestock, and rabbits. *Y. enterocolitica* is transmitted by ingestion of contaminated food (often milk and pork) and water, probably by the fecal-oral route, and perhaps by contact with infected animals.

Clinical Manifestations

Y. enterocolitica is a significant and invasive enteric pathogen that causes several well-recognized diseases, especially in younger persons, and several uncommon postinfection syndromes (11). The most common diseases caused by the bacterium are (hemorrhagic) enterocolitis, terminal ileitis, mesenteric lymphadenitis (pseudoappendicular syndrome), septicemia, and focal infections in many extraintestinal sites.

Enterocolitis caused by *Y. enterocolitica* is characterized by diarrhea, low fever, and abdominal pain. Leukocytes

and, often, blood can be present in stools. The illness usually is self-limiting, but many complications have been reported (11). Patients (commonly teenagers) with pseudoappendicular syndrome usually present with fever, right lower quadrant tenderness, and abdominal pain. The syndrome is clinically similar to and often misdiagnosed as appendicitis but is actually mesenteric lymphadenitis and terminal ileitis. *Y. enterocolitica* can be cultured from the lymph nodes and ileum. Many cases of fatal bacteremia and septic shock have been associated with *Y. enterocolitica*-contaminated blood transfusion products (9, 26).

Biogroups and Serotypes

Most isolates can be serotyped as one of >50 serotypes according to somatic O antigens. Only certain biotypes and serotypes of *Y. enterocolitica* are generally considered enteric and invasive pathogens. In the United States, serotypes O:8 and O:5,27 cause mesenteric lymphadenitis and the most invasive forms of *Y. enterocolitica* disease.

Isolation

Although strains of *Y. enterocolitica* grow faster at 37 than at 25°C, the lower temperature is recommended for primary isolation. Strains of *Y. enterocolitica* grow well on MacConkey agar incubated at 37°C, but the colonies are much smaller than those of other species of *Enterobacteriaceae*. Cefsulodin-irgasan-novobiocin (CIN) agar is specifically designed to isolate *Yersinia* spp. Cultures on CIN agar are incubated at 32°C for 24 h or at 22 to 25°C for 48 h. After 18 to 24 h on CIN agar, colonies of *Y. enterocolitica* are translucent without dark red centers; by 48 h, the colonies are dark pink with translucent borders and are occasionally surrounded by a zone of precipitated bile. Both pathogenic and nonpathogenic strains of *Y. enterocolitica* and other *Yersinia* species grow on CIN agar, but most other bacteria (except *Citrobacter* spp.) are inhibited. Strains of *Y. enterocolitica* usually are lactose negative, but lactose-positive strains exist.

If a dedicated medium for *Yersinia* spp. is not used, cultures on MacConkey agar can be examined after 24 h at 35 to 37°C for small colorless colonies that become much larger after an additional 24 h of incubation at room temperature.

Many laboratories culture for *Y. enterocolitica* only on request, because the routine use of special selective media for *Y. enterocolitica* (CIN agar) is a low-yield procedure and is not cost-effective.

Identification

On TSI agar, most isolates of *Y. enterocolitica* typically produce an acid/acid reaction with no gas or H_2S. Some strains produce an alkaline reaction because of slow sucrose fermentation or the production of alkaline products from peptones in the medium. The typical acid/acid reaction on TSI agar is the same as that of some *Enterobacteriaceae* such as *E. coli*; therefore, *Y. enterocolitica* can be missed if TSI agar is used to screen for *Salmonella* and *Shigella* spp. On Kligler's iron agar, *Y. enterocolitica* will produce an alkaline/acid reaction similar to that of *Salmonella* and *Shigella* spp. Most isolates of *Y. enterocolitica* are urea positive (often at 24 h; sometimes several days are required) and motile at 25 but not 37°C.

Serodiagnosis

In culture-negative cases of suspected *Y. enterocolitica* or *Y. pseudotuberculosis* disease, the results of serologic assays (microhemagglutination, complement fixation, and enzyme immunoassay) for antibody to these bacteria might be helpful. Such assays are available in some large commercial clinical laboratories.

Y. pseudotuberculosis

Y. pseudotuberculosis is biochemically similar to *Y. pestis*, causes illnesses (usually in persons 5 to 15 years old) similar to those caused by *Y. enterocolitica*, and is isolated (usually from blood) only rarely in the United States. The disease caused by this bacterium is a zoonosis. The natural reservoirs of the bacterium are rodents, wild animals, and game birds throughout the world.

Other Species of *Yersinia*

The other eight species of *Yersinia* (*Yersinia frederiksenii, Yersinia intermedia, Yersinia kristensenii, Yersinia aldovae, Yersinia bercovieri, Yersinia mollaretti, Yersinia rohdei,* and "*Yersinia ruckeri*" are also found in both intestinal and extraintestinal specimens and are biochemically similar to each other. These species can grow at 4°C and on CIN agar, and they can multiply in refrigerated foods. The pathogenic potential of these eight species is controversial, but at this time, isolates should not be disregarded.

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Candida, Cryptococcus, and Other Yeasts of Medical Importance

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NATURAL HABITAT AND CLINICAL SIGNIFICANCE

Yeasts are ubiquitous in our environment, being found on fruits, vegetables, and other plant materials (exogenous). Some live as normal inhabitants in and on our bodies (endogenous). Therefore, they may be found in specimens without having any clinical significance. Yeasts are considered opportunistic pathogens and as such may be cultured from specimens of patients debilitated in some fashion, e.g., by hormone imbalance; by the administration of immunosuppressive agents such as corticosteroids, anticancer drugs, and the newer anti-AIDS drugs; or by the overuse of broad-spectrum antibiotics. Patients who undergo transplant surgery and the concomitant administration of immunosuppressive drugs often acquire yeast infections. Diseases such as AIDS and others that cause a diminution or depletion of the immunological system are also predisposing factors for yeast infections. Endocarditis caused by yeasts has been associated with the use of nonsterile equipment by drug addicts. Fungemia may occur when indwelling catheters are not changed or removed at frequent intervals; it also has been reported in infants supported by lipid-supplemented hyperalimentation (17).

Yeasts are by far the most common fungi isolated from human patients. The decision as to the significance of their presence in a specimen ultimately rests with the physician, but accurate, complete information from the laboratory is essential for reaching a reasonable conclusion. Type of specimen (e.g., closed, normally sterile sites rather than sputum or urine), number of specimens positive with the same organism from the same patient, and number of colonies formed are all critical pieces of information and should be carefully noted.

CHARACTERISTICS OF YEASTS

Yeasts are unicellular, eukaryotic, budding cells that are generally round to oval or, less often, elongate or irregular in shape. They multiply principally by the production of blastoconidia (buds). When blastoconidia are produced one after the other in a linear fashion without separation, a structure termed a pseudohypha is formed. Under certain circumstances, some yeasts may produce true septate hyphae. Such circumstances are associated with the diminution of oxygen, e.g., in host tissues or for submerged colonies in agar medium, at the bottom of broth medium in a test tube, or in the presence of 5 to 10% CO_2.

Cultures of yeasts are moist, creamy, or glabrous to membranous in texture. Several produce a capsule that may make the colony mucoid. With rare exceptions, aerial hyphae are not produced. Colonies may be hyaline, brightly colored, or darkly pigmented because of the presence of melanins. Organisms with darkly pigmented colonies, found in the family Dematiaceae, are discussed in chapter 67 of this Manual. Dimorphic fungal pathogens possessing a yeast phase in tissue are also discussed in other chapters.

Although there are many yeast genera and hundreds of yeast species, relatively few of these produce disease in humans and animals. Yeasts generally are identified (classified) by observing the macroscopic and microscopic features mentioned above. Usually, biochemical tests are also required for definitive identification to species level. In most instances, the pathogenic yeasts are found among the *Deuteromycetes*, or Fungi Imperfecti, that is, fungi that do not exhibit the sexual or teleomorphic state in culture.

Yeasts also may be classified as *Ascomycetes* or *Heterobasidiomycetes* depending on their method of sexual reproduction. A few genera may produce the sexual state on standard mycological media over time. In some instances, it may be necessary to identify the teleomorphic state of a yeast culture. This is best accomplished by submitting the culture to a reference laboratory for further study.

DIRECT EXAMINATION

The appropriate examination of a clinical specimen is essential prior to proper processing of the material. Additionally, examination often will aid the laboratorian and the physician in a preliminary identification, either ruling in or ruling out certain pathogenic yeasts. Certain methods are universal to the preliminary observation of fungi in a specimen, e.g., Gram stain, calcofluor, and 20% KOH. However, others, such as India ink preparation for the demonstration of a capsule, are used for the yeasts and yeast-like fungi only. This preparation is used with cultures and on specimens of urine, cerebrospinal fluid, etc., that have been centrifuged. It generally is not useful on primary specimens such as sputum or on other materials that do not allow even distribution of the ink. It must be remembered that if 20%

Aspergillus, Fusarium, and Other Opportunistic Moniliaceous Fungi

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The incidence of infections caused by moniliaceous fungi that typically exist as ubiquitous saprophytic and plant parasitic moulds has risen dramatically in recent years (42). The increased use of antimicrobial agents and more aggressive and prolonged chemotherapeutic regimens for treatment of malignant diseases have contributed to this trend (7, 62, 113). Similarly, the advent of routine organ transplantation and implantation of prosthetic devices, the introduction of highly technical methods for advanced life support, and the management of newer diseases, especially AIDS, and physiologic disorders have put patients at greater risk of infection by fungi historically thought of as "laboratory contaminants" (54, 140). Moreover, as patient longevity increases with better management of AIDS or neoplasmas, there is greater risk for infection by uncommon fungal pathogens (42, 124). This chapter focuses on those fungi that grow in tissue in the form of hyaline or lightly colored septate hyphal elements and that produce a varied clinical spectrum.

CLINICAL MANIFESTATIONS AND DISEASE TERMINOLOGY

The spectrum of disease caused by hyaline moulds is diverse, is largely determined by the local and general immunologic and physiologic state of the host, and may be symptomatic or asymptomatic. In most instances, the portal of entry for fungal propagules is either through a break in the epidermis or by way of the lungs. Noted exceptions to this include introduction into the body by means of contaminated surgical instruments, intraocular lens, and prosthetic devices or other contaminated materials or solutions associated with surgery and/or routine health care (90, 136, 140).

Although these opportunistic moulds grow in most body tissues and fluids, colonization or invasion is commonly, but not solely, associated with subcutaneous soft tissue and mucous membranes. Individuals whose resistance is lowered as a result of a severe debilitating disease or immunosuppressive therapy typically suffer from invasive pulmonary or paranasal sinus infection, but in some instances, the infecting fungus may spread to surrounding tissues or disseminate to virtually any organ (90, 113, 136). Noninvasive forms of infection also have been noted in debilitated individuals as well as in individuals with apparently normal defense mechanisms. In such cases, the fungus colonizes a preexisting cavity in the lungs such as an ectatic bronchus, a tuberculous cavity, or a lung cyst (20). Other clinical syndromes are also recognized. Infections of the ear canal, nail, cutaneous and subcutaneous tissues, cornea, and other sites manifesting as cellulitis, bursitis, nephritis, endocarditis, peritonitis, fungemia, and, rarely, cerebral infections and involvement of the central nervous system have been documented (8, 121).

The use of the term aspergillosis to define infections caused by species of *Aspergillus* is well established, but the practice of coining disease names based on the genus of the fungus involved has disadvantages for infections caused by uncommon or rare fungal pathogens. The wide variety of fungi involved has made it difficult to place the organisms into accessible groups, and problems arise when fungus names are changed. To avoid the coining and proliferation of unnecessary new disease names based on the genus of the fungus involved, two major disease groups have been proposed: hyalohyphomycosis and phaeohyphomycosis (2, 3, 105). Each group encompasses a variety of clinical syndromes but is based on the presence of septate hyphal elements without or with pigmentation or melanin in the cell wall (2, 3, 105). Phaeohyphomycosis, as originally defined, is a heterologous group of infections characterized by the presence of dark (brown to black)-pigmented fungal elements in tissue (see chapter 67 of this Manual); however, there has been some confusion about the use of the term. If melanin pigmentation in tissue is the primary criterion for inclusion under the broad umbrella of this mycosis, several fungi that are dematiaceous in vitro should be excluded from this group, since they grow as hyaline elements in tissue. Species of *Scedosporium*, for instance, form pigmented conidia in vitro and hyaline hyphae in tissue that are indistinguishable from those of *Aspergillus* species. Another example of this anomaly is the strongly pigmented fungus *Nattrassia mangiferae* (*Scytalidium dimidiatum*), which usually forms hyaline or lightly pigmented hyphal elements in tissue. Although special stains (e.g., the Masson-Fontana silver stain) may help detect melanin in fungal elements in tissue, the results are not always decisive. Some fungi with variable pigmentation, such as *Sporothrix schenckii*, may stain faintly or inconsistently (91). Recently, a subcommittee of the International Society for Human and Animal Mycology (ISHAM) added to the confusion by

defining phaeohyphomycosis as infection caused by a dematiaceous fungus (117). The term "hyalohyphomycosis" was proposed as a complement to phaeohyphomycosis for all subcutaneous and systemic infections in which the fungi involved have a tissue form consisting of hyaline, septate, branched, and, occasionally, toruloid mycelial elements (121). Although it was not intended to replace disease names such as aspergillosis, the term received qualified endorsement by the ISHAM subcommittee (117) only as a general term for infections caused by "*unusual* hyaline pathogens." This committee recommended that, rather than "generic" disease names, fungal diseases be given names that provide a specific description of the pathology and name the causative agent, e.g., subcutaneous cyst caused by fungus X. This approach may resolve the current difficulties in trying to categorize infections by the color of fungal elements in tissue.

ETIOLOGIC AGENTS

The opportunistic hyaline moulds that have been reported to cause infection in humans and animals are listed in Table 1. Verification of authenticity of reports can be difficult if the fungus is inadequately described and illustrated and if representative cultures are not maintained in culture collections. This chapter describes the more commonly occurring opportunists and briefly comments on some rarer agents.

Classification and Morphology

The opportunistic hyaline moulds are distributed throughout the kingdom Fungi and belong to genera of the Ascomycotina, Basidiomycotina, and Fungi Imperfecti. The Fungi Imperfecti is a special division or "form" division consisting of fungi for which no sexual stage (teleomorph) is known and fungi that are the asexual stages (anamorphs) of ascomycetes and basidiomycetes. Today, the teleomorphs of many of the opportunistic fungi are known. Although teleomorphs occasionally are isolated in culture, they may be difficult to obtain by routine culture methods. The Fungi Imperfecti includes both yeasts (unicellular growth) and moulds (filamentous growth) and is divided into three form-classes: (i) Blastomycetes, including unicellular organisms reproducing by budding or fission and sometimes producing hyphae (yeasts and yeastlike fungi; see chapter 61 of this Manual); (ii) Hyphomycetes; and (iii) Coelomycetes. Reproduction in mould fungi is characterized by the production of conidia, which are reproductive propagules produced following mitotic division of the nucleus. Conidia are formed when the nucleus migrates into the propagule from a specialized cell called a conidiogenous cell. The conidiogenous cell(s) may be borne on an erect simple or branched structure known as a conidiophore (Hyphomycetes) or within a specialized fruiting body called a conidioma (Coelomycetes).

Most of the pathogenic moulds are classified as Hyphomycetes. Identification of Hyphomycetes is based on morphology of the conidia and the mechanisms by which conidia are formed (conidiogenesis). Three basic tools are necessary for practical observation of these features. (i) An ocular micrometer (see chapter 67) is essential for recording the sizes of conidia or of sexual spores when present. Identification of the less common opportunists requires comparison with published taxonomic descriptions in which size is often a key criterion for species distinction. (ii) A dissecting microscope with magnification up to ×60 and basal illumination is useful for examining petri plate cultures or culture tubes for the presence of conidia in chains or slimy heads, specialized structures such as hülle cells, sclerotia, conidiomata, or sexual fruiting bodies forming under the aerial mycelium or embedded in the agar. (iii) Microscopic mounts that allow observation of how a fungus forms its conidia are also necessary. Slide culture preparations are excellent for many fungi (155), but conidial structures of some species of *Aspergillus* or *Penicillium* may not be typical in slide culture conditions.

Morphologic features of importance for identification of conidial fungi include (i) conidium size, shape, and pattern of septation; (ii) color of conidia and conidiophore, whether light (hyaline or moniliaceous) or dark (dematiaceous); (iii) developmental aspects of conidiogenesis, including nature of the conidiogenous cell; (iv) mechanism of conidium liberation or dehiscence; and (v) structure of the conidioma (if present). Differences in conidial shape and septation, which distinguish groupings known today as Saccardo spore groups (119), are readily observable characteristics convenient for preliminary distinction. Conidia may be single celled (amerosporae) or have one (didymosporae) or more (phragmosporae) septa. If a fungus produces both nonseptate and septate conidia, the conidia are often referred to as micro- and macroconidia. Conidia also vary in shape, being long and narrow (scolecosporae; as in *Fusarium* species), spirally coiled (helicosporae), or star shaped (staurosporae). Development of a conidium may occur by conversion of an existing cell or several cells (thallic-arthric) or may involve new wall building or blowing out of a portion of the wall (blastic). Conidiogenesis usually occurs at a particular location on a conidiogenous cell. If development occurs at a site that remains fixed and gives rise to more than one conidium, then the site is stable or determinate. If development occurs at new points on the conidiogenous cell (or axis), then the site is unstable or indeterminate. New sites of conidial development may occur as the axis lengthens (progressive) or shortens (retrogressive). The conidiogenous cell may produce only a single conidium or multiple conidia. Sympodial development involves the development of a single conidium at successive sites on a lengthening axis. Conidiogenous cells that are specialized to produce multiple conidia include the phialide and the annellide. Although it is often difficult to distinguish between these two types of cells, the annellide elongates, and sometimes narrows, during the formation of each new conidium, leaving an often imperceptible series of rings or scars on the conidiogenous cell. Scrutiny of the cell under an oil immersion objective may be necessary to make this distinction. Phialides and annellides produce conidia that accumulate either in slimy masses or in chains with the youngest at the base of the chain (basipetal). Conidia may also form in acropetal chains, with the youngest conidium at the tip of the chain. Distinction between acropetal and basipetal chains is possible by careful scrutiny of the size and wall morphology of the top and bottom conidia of the chain. The youngest conidium is recognizable by its smaller size, lighter color if the conidia are pigmented, and differences in wall ornamentation if the conidia are roughened. Some conidiogenous cells are specialized to simultaneously form multiple conidia over the surface of the swollen cell. When mature, conidia detach by fission of a double septum (schizolytic dehiscence) or by sacrifice of a supporting cell (rhexolytic dehiscence) either by fracture of a thin-walled region of the supporting cell or by lysis. Lytic dehiscence